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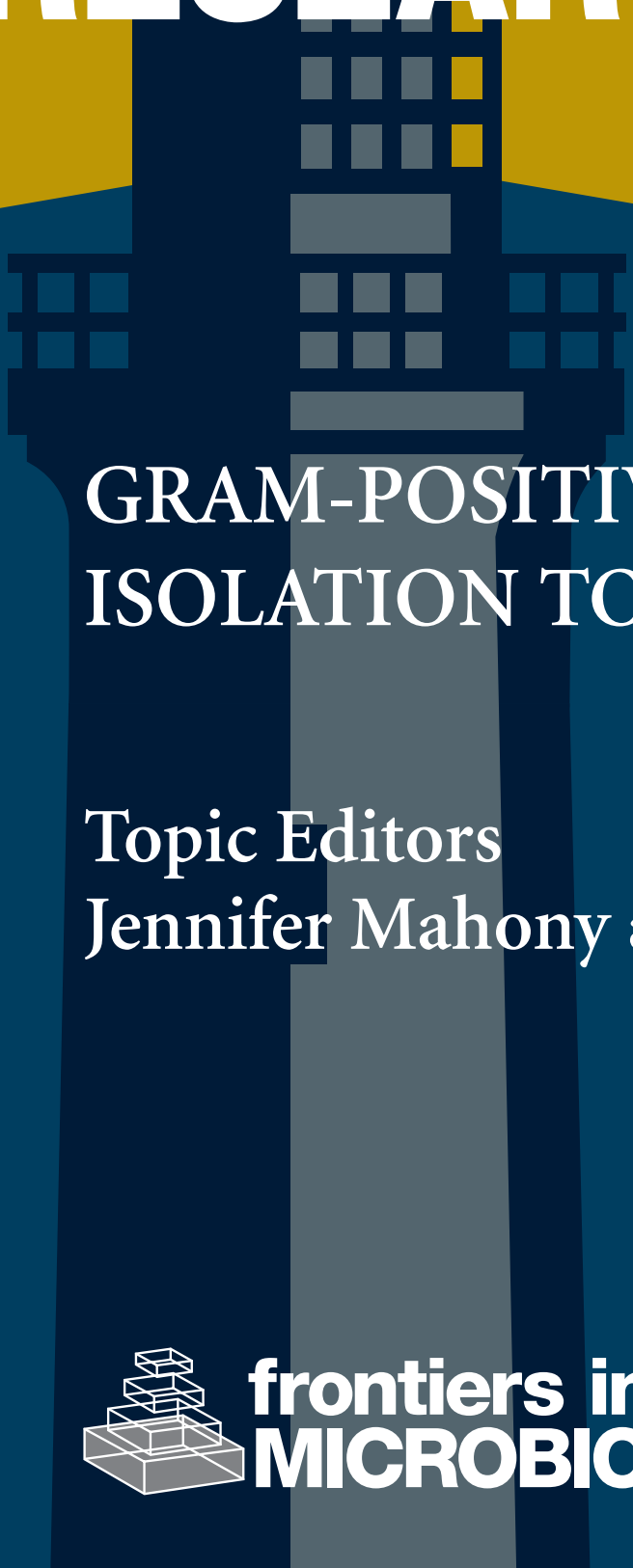

GRAM-POSITIVE PHAGES: FROM ISOLATION TO APPLICATION

Topic Editors
Jennifer Mahony and Douwe van Sinderen

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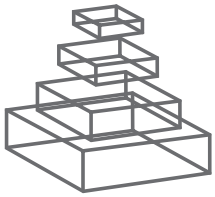

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Cover image provided by Ibbl sarl, Lausanne CH

ISSN 1664-8714
ISBN 978-2-88919-493-3
DOI 10.3389/978-2-88919-493-3

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GRAM-POSITIVE PHAGES: FROM ISOLATION TO APPLICATION

Topic Editors:
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Phage biology is one of the most significant and fundamental aspects of biological research and is often used as a platform for model studies relating to more complex biological entities. For this reason, phage biology has enjoyed focused attention and significant advances have been made in the areas of phage genomics, transcriptomics and the development and characterisation of phage-resistance mechanisms. In recent years, considerable research has been performed to increase our understanding of the interactions of these phages with their hosts using genomic, biochemical and structural approaches. Such multidisciplinary approaches are core to developing a full understanding of the processes that govern phage infection, information that may be harnessed to develop anti-phage strategies that may be applied in food fermentations or applied in a positive sense in phage therapy applications. The co-evolutionary processes of these phages and their hosts have also been a considerable focus of research in recent years. Such data has promoted a deeper understanding of the means by which these phages attach to and infect their hosts and permitted the development of effective anti-phage strategies. Furthermore, the presence and activity of host-encoded phage-resistance systems that operate at various stages of the phage cycle and the potential for the application of such systems consolidates the value of research in this area. Conversely, phages and their components have been applied as therapeutic agents against a number of pathogens including, among others, Clostridium difficile, Lactococcus garviae, Mycobacterium spp., Listeria spp. and the possibilities and limitations of these systems will be explored in this topic. Additionally, phage therapeutic approaches have been applied to the prevention of development of food spoilage organisms in the brewing and beverage sectors and exhonorate the positive applications of phages in the industrial setting. This research topic is aimed to address the most current issues as well as the most recent advances in the research of phages infecting Gram-positive bacteria covering areas such as phages in food fermentations, their impact in industry, phage ecology, genomics, evolution, structural analysis, phage-host interactions and the application of phages and components thereof as therapeutic agents against human and animal pathogens.

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frontiers in
MICROBIOLOGY

EDITORIAL
published: 11 February 2015
doi: 10.3389/fmicb.2015.00061

Gram-positive phage-host interactions

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Keywords: interactions, lactic acid bacteria, phage attachment, host receptor, dairy, phage therapy

Bacteriophage research has seen many peaks and troughs over the past century ascending with phage therapy and application in the early 1900's; a research peak which was largely overshadowed by the dawning of the antibiotic era, and which has now deservedly regained attention as an approach against the problematic rise in antibiotic-resistant pathogenic bacteria. Following this initial scientific highlight, the advent of molecular biology and biotechnology sparked a renewed interest in phages and their encoded enzymes and promoters, which are still employed as research tools today. Much of this research was conducted using phages of Gram-negative bacteria, particularly *Escherichia coli*, due to the reliability of the host and the ease of protein (over) production, in particular many enzymes, in a compatible host background. Consequently, coliphages such as T4 and lambda served as model phages in the development of molecular tools and the fundamental understanding of phage-host interactions. The advent of new generation sequencing technologies has in recent years provided a vast array of sequence data relating to Gram-positive phages and their hosts, which in turn has permitted the development of analogies between Gram-negative and Gram-positive phages. For example, sequence analysis of *Bacillus subtilis* and *Lactococcus lactis* phages SPP1 and Tuc2009, respectively, revealed genomes with a conserved gene and/or functional order relative to lambda, the main model for *Siphoviridae* phages. While the Gram-negative models have been extremely useful platforms, many questions have remained unanswered owing to the fundamental structural and compositional differences between the cell walls of Gram-negative and positive cells. In response to this knowledge gap, there has been a significant upsurge in research in the area of phages infecting Gram-positive bacteria and in particular, lactococcal phage-host interactions, which have now become one of the leading model systems along with the above-mentioned *Bacillus subtilis* phage SPP1 and the mycobacteriophage L5.

In the ensuing 11 articles, many key advances that now define our understanding of phage-host interactions of Gram-positive bacteria and their infecting phages are described. We collate these advances and define the current knowledge of cell wall structures that present the target molecule of phage attachment (Munsch-Alatossava and Alatossava, 2013; Chapot-Chartier, 2014) and the phage-encoded adhesion complexes that phage employ to attach to their host in lactococci (Spinelli et al., 2014). Additionally, we explore the role of genomics in advancing knowledge on phages infecting previously underrepresented bacterial species that are of practical relevance to the food industry including the *Leuconostoc, Oenococcus* and *Weissella* (Kot et al., 2014; Mahony and van Sinderen, 2014), and phage therapy including *Listeria* and *Clostridium* spp. (Hagens and Loessner, 2014; Hargreaves and Clokie, 2014; Ly-Chatain, 2014). Furthermore, the research articles reinforce the continuing need for isolation and characterisation of phage isolates to retain a current perspective on the ever-changing phage genomics landscape (Cavanagh et al., 2014) and the possibility of deriving and understanding anti-phage measures that may be harnessed in various biotechnology sectors, in particular the dairy industry (Ali et al., 2014; Chirico et al., 2014).

ACKNOWLEDGMENTS

Jennifer Mahony is in receipt of a Technology Innovation Development Award (TIDA) (Ref. No. 14/TIDA/2287) funded by Science Foundation Ireland (SFI). Douwe van Sinderen is supported by a Principal Investigator award (Ref. No. 13/IA/1953) through SFI.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 January 2015; accepted: 18 January 2015; published online: 11 February 2015.

Citation: Mahony J and van Sinderen D (2015) Gram-positive phage-host interactions. Front. Microbiol. ***6****:61. doi: 10.3389/fmicb.2015.00061*

This article was submitted to Virology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 24 January 2014
doi: 10.3389/fmicb.2014.00007

Current taxonomy of phages infecting lactic acid bacteria

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Phages infecting lactic acid bacteria have been the focus of significant research attention over the past three decades. Through the isolation and characterization of hundreds of phage isolates, it has been possible to classify phages of the dairy starter and adjunct bacteria *Lactococcus lactis, Streptococcus thermophilus, Leuconostoc* spp., and *Lactobacillus* spp. Among these, phages of *L. lactis* have been most thoroughly scrutinized and serve as an excellent model system to address issues that arise when attempting taxonomic classification of phages infecting other LAB species. Here, we present an overview of the current taxonomy of phages infecting LAB genera of industrial significance, the methods employed in these taxonomic efforts and how these may be employed for the taxonomy of phages of currently underrepresented and emerging phage species.

Keywords: *Lactococcus, Streptococcus, Lactobacillus,* dairy, food fermentation, genetics

INTRODUCTION

The lactic acid bacteria (LAB) are a heterogeneous group of Gram positive, non-spore-forming bacteria with a rod-shaped or coccoid morphology. As their name suggests, lactic acid is the predominant end-product when LAB engage in hexose fermentation, and it is due to the pre-servative and palatable properties of lactic acid that has for many centuries rendered this group of bacteria applicable in food and feed fermentations, in particular for the production of dairy products. Strains of *Lactococcus lactis* and *Streptococcus thermophilus* are the most intensely employed starter bacteria in the dairy fermentation industry globally (Deveau et al., 2006), while strains of *Lactobacillus* spp. and *Leuconostoc* spp. are widely used as adjuncts in such processes (Nieto-Arribas et al., 2010). Furthermore, in vegetable fermentations, ecological studies have reported the complex and evolving microbial landscape with strains of *Lactobacillus*, *Pediococcus, Leuconostoc* and *Weisella* spp. implicated at various stages of the fermentation (Lu et al., 2003, 2012). However, as with most living organisms, LAB are susceptible to viral infection by (bacterio) phages, which may impact on the quality, flavor and texture of the final product. The application of these bacteria in modern fermentation processes involves intensive production and throughput, thereby increasing the risk of bacteriophage infection. Phages are particularly problematic in fermentation systems that repeatedly use the same cultures or culture mixes/rotations as phages are known to persist in the processing environs until a suitable host is available to infect. Consequently, phages of LAB have enjoyed significant attention, particularly over the past three decades. All LAB-infecting phages belong *Caudovirales* order and most of them to the *Siphoviridae* family that possess long non-contractile tails and isometric or prolate capsids (Mahony et al., 2012a). Additionally, phages with short non-contractile tails (*Podoviridae*) and those displaying long contractile tails (*Myoviridae*) have also been described for some LAB genera (Chibani-Chennoufi et al., 2004; Chopin et al., 2007; Deasy et al., 2011). Undoubtedly, the most intensely researched LAB-infecting phages are those of the dairy starter bacteria *L. lactis* and *S. thermophilus* (Neve et al., 1998; Lucchini et al., 1999; Quiberoni et al., 2000; Brussow and Desiere, 2001; Proux et al., 2002; Mahony et al., 2006; Guglielmotti et al., 2009; Rousseau and Moineau, 2009; Collins et al., 2013). In recent years, genome sequencing technologies have improved and diversified drastically, and this has probably been the single greatest driving force behind the acquisition of current data regarding LAB-infecting phage biodiversity, taxonomy and evolution. Current phage taxonomic efforts significantly depend on comparative genomic analysis and derived information. Phage taxonomy is a contentious issue, yet a highly important one since such classifications are core to the development of detection tools and prevention and control measures. Here, we will review the changing face of LAB phage taxonomy, the major advances to date and how such taxonomic efforts may influence future efforts at minimizing the risk of phage infection.

LACTOCOCCAL PHAGES

Phages that infect host strains with resident prophages and/or phage-resistance systems are subject to significant genome rearrangements, which appears to be a major evolutionary driving force among such phages (Labrie and Moineau, 2007). Therefore, it is of great significance that the genome sequences of a number of lactococcal strains and their resident prophages have become available to understand the dynamic processes that may lead to such genome rearrangements (Chopin et al., 2001; Ventura et al., 2007; Wegmann et al., 2007; Siezen et al., 2010; Ainsworth

et al., 2013; Du et al., 2013). *L. lactis* strains employed in the dairy industry belong to one of two subspecies, namely *L. lactis* ssp. *lactis* or *L. lactis* ssp. *cremoris*. While lactococcal strain diversity may be limited, their infecting phages have proven their genomic elasticity and evolutionary capabilities in order to survive and evade hygiene measures, processing conditions and host-encoded phage-resistance mechanisms (McGrath et al., 1999; Scaltriti et al., 2010; Samson et al., 2013). To a large degree, this co-evolution, coupled to the intensity of production, has supported the ever-increasing genetic diversity of these phages as we currently recognize and classify them.

Phages of *L. lactis* were first classified in 1984 into four groups based on morphology, serological reactions and DNA-DNA hybridization of 25 phages (Jarvis, 1984). This study was the basis of further classifications of lactococcal phages resulting in the identification of dominant species in isolation studies and furthermore the identification of rarely encountered and emerging species (Prevots et al., 1990).

In 1991, this classification was updated and 12 species were identified based on DNA homology and morphology (Jarvis et al., 1991). The virion morphologies were identified as belonging to one of two families i.e., *Siphoviridae* and *Podoviridae*. In 2002, the lactococcal phage BK5-t was proven to be a member of the polythetic P335 species, which has both lytic and temperate members (Labrie and Moineau, 2002), thus reducing the number of lactococcal phage species to eleven.

Most recently, in 2006, Deveau and colleagues reassessed existing phage isolates of *L. lactis* and reduced the number of currently existing lactococcal phage species to ten (Deveau et al., 2006). This re-classification highlighted the extinction of the P107 species and the amalgamation of BK5-t, 1483 and T187 in the P335 species (Deveau et al., 2006). Furthermore, it also highlighted the emergence of new species, such as the Q54 and 1706 species, which were previously unknown or unclassified (Deveau et al., 2006). Over the past decade, representative members of the rare and emerging lactococcal phage species, 949 (Samson and Moineau, 2010), P087 (Villion et al., 2009), P034 (Kotsonis et al., 2008), Q54 (Fortier et al., 2006), 1358 (Dupuis and Moineau, 2010), KSY1 (Chopin et al., 2007) and 1706 species (Garneau et al., 2008), have been sequenced and providing essential information to corroborate this classification scheme.

The above taxonomic studies have all compounded the necessity of combining taxonomic methods (including electron microscopy, DNA-DNA hybridizations/genome sequencing) that complement each other and provide an effective means for grouping phages (Jarvis, 1984; Jarvis et al., 1991; Deveau et al., 2006). In 1990, Prevots and colleagues identified that the virulent 936 species dominated their collection of 101 phage isolates (Prevots et al., 1990) and from information gathered over the ensuing 23 years, this dominance has been retained (Deveau et al., 2006; Rousseau and Moineau, 2009; Castro-Nallar et al., 2012; Murphy et al., 2013). The genome architecture and content of the 936 phages is highly conserved and the success of this species may be attributed to the limited number of strains available to the dairy industry, permitting their propagation and evolution (Mahony et al., 2012b). The P335 phage species is currently the second most frequently isolated species in the dairy industry and this represents a genetically diverse group of phages that may be lytic or temperate (Mahony et al., 2013). Correlating with their industrial significance, the 936 and P335 species phages also dominate in terms of fundamental research pertaining to their genomics and phage-host interactions and serve as models for phages of a variety of Gram positive bacterial hosts (Veesler et al., 2012; Bebeacua et al., 2013; Collins et al., 2013). To date, in excess of 70 lactococcal phage genomes have been sequenced to completion with approximately 70% of these belonging to the 936 species according to the EMBL-EBI website at the time of writing (www.ebi.ac.uk/genomes/phage.html) (**Table 1**). Given the lack of complexity of most lactococcal starter cultures, it is not surprising that these species continue to dominate and evolve, however, there are ample possibilities for genetic rearrangements and development of novel species as has been observed in the case of the Q54 species (Fortier et al., 2006). The emergence of such novel species highlights the necessity of regular revisions of the taxonomy of these phages. Furthermore, while the small, isometric-headed phages are the most abundant morphotype of lactococcal phages, the observation of lactococcal siphophages with unusually long tails (949 species), or *Podoviridae* with decorated capsid structures (KSY1), indicates that morphological assessment remains a useful tool in the taxonomic characterization of such phages as a complement to genotyping.

S. thermophilus PHAGES

In contrast to the phages of *L. lactis*, all phages infecting *S. thermophilus* display a similar morphology with long, non-contractile tails (typically more than 200 nm in length) and isometric capsid structures, thus belonging to the *Siphoviridae* family (Brussow et al., 1994; Bruttin et al., 1997; Levesque et al., 2005; Guglielmotti et al., 2009; Zinno et al., 2010; Mills et al., 2011). Therefore, electron microscopy and associated morphological analysis provides little scope for differentiation between these phages, thus necessitating the application of other methods of discernment.

In 1994, host range and serological reaction analysis of 81 phages infecting *S. thermophilus* directed the first significant classification of these phages into four classes (Brussow et al., 1994). Only a few years later in 1997, a refinement of this classification was determined through the combined application of DNA restriction profiling, structural protein profiling and host range analysis defined that these phages should be classified into two major groups (Le Marrec et al., 1997). These groups were accordingly named the *cos* (cohesive ends) and *pac* (headful packaging method) groups, in congruence with their mode of DNA packaging. This taxonomic system was upheld until the recent isolation of phage 5093, which infects the Mozzarella starter strain CSK939 (Mills et al., 2011). The genome of this phage was sequenced and revealed a novel genotype among *S. thermophilus* phages. It possesses greater homology to non-dairy streptococcal prophage sequences than to the genomes of sequenced *S. thermophilus* phages. This singular phage represents the newest addition to the lactic streptococcal phage taxonomic grouping system and, as yet, remains the only known member of this third species of *S. thermophilus* phages (**Table 1**). Furthermore, morphological analysis of this phage revealed globular structures at the tail tip region, a novel feature among lactic streptococcal

Table 1 | Current taxonomy of LAB phages with sequenced members.

Host	Phage family	Phage species	No. of fully sequenced members	Taxonomy reference(s)
L. lactis	*Siphoviridae*	936	51	Deveau et al., 2006
		P335	15	Deveau et al., 2006
		c2	2	Deveau et al., 2006
		1358	1	Deveau et al., 2006
		Q54	1	Deveau et al., 2006
		P087	1	Deveau et al., 2006
		1706	1	Deveau et al., 2006
		949	2	Deveau et al., 2006
	Podoviridae	P034	1	Deveau et al., 2006
		KSY1	1	Deveau et al., 2006
S. thermophilus	*Siphoviridae*	*cos*	6	Le Marrec et al., 1997
		pac	6	Le Marrec et al., 1997
		5093-like	1	Mills et al., 2011
Ln. mesenteroides	*Siphoviridae*	Group Ia and b	2	Ali et al., 2013
Ln. peudomesenteroides	*Siphoviridae*	Group IIa–d	2	Ali et al., 2013
Lb. brevis	*Myoviridae*	Unnamed	1	Deasy et al., 2011; Jang et al., 2011
Lb. casei	*Siphoviridae*	Unnamed	1	Villion and Moineau, 2009
Lb. delbrueckii	*Siphoviridae*	Unnamed	6	Villion and Moineau, 2009
Lb. fermentum	*Siphoviridae*	Unnamed	2	Yoon and Chang, 2011; Zhang et al., 2011
Lb. gasseri	*Siphoviridae*	Unnamed	1	Villion and Moineau, 2009
	Myoviridae	Unnamed	1	Villion and Moineau, 2009
Lb. helveticus	*Myoviridae*	Unnamed	1	Zago et al., 2013
Lb. paracasei	*Siphoviridae*	Unnamed	2	Villion and Moineau, 2009
	Myoviridae	Unnamed	1	Alemayehu et al., 2009
Lb. plantarum	*Siphoviridae*	Unnamed	5	Villion and Moineau, 2009
	Myoviridae	Unnamed	1	Villion and Moineau, 2009
Lb. rhamnosus	*Siphoviridae*	Unnamed	1	Villion and Moineau, 2009
Lb. sanfranciscensis	*Siphoviridae*	Unnamed	1	Ehrmann et al., 2013

phages, again reinforcing the application of morphological assessment of phage isolates in parallel with other characterization tools.

Leuconostoc PHAGES

Leuconostoc spp. are part of undefined composite starter mixes of many semi-hard cheeses and are required for aroma and flavor formation in such cheeses (Cogan and Jordan, 1994). Phages of *Leuconostoc* spp. have received growing and deserved attention in recent years in terms of phage isolation studies and genomic analysis pertaining to vegetable and dairy fermentations (Sutherland et al., 1994; Gindreau et al., 1997; Greer et al., 2007; Lu et al., 2010; Kleppen et al., 2012; Ali et al., 2013; Kot et al., 2013). With respect to those infecting dairy starter and adjunct strains of *Leuconostoc mesenteroides* and *pseudomesenteroides*, the most significant taxonomic classification has been provided this year following the analysis of 83 phages by host range, morphology and DNA homology (Ali et al., 2013). This resulted in the identification of species-specific groups capable of infecting one species of *Leuconostoc* (**Table 1**). The phages were primarily grouped into two major classes based on their non-overlapping host ranges, I and II (i.e., those capable of infecting either *Ln. mesenteroides* or *Ln. pseudomesenteroides* strains). All phages were observed to possess long non-contractile tails and isometric capsids, consistent with the features of *Siphoviridae* phages but with distinct baseplate appendages at their tail tip regions. In the case of *Ln. mesenteroides* (group I), one dominant species of phages with globular appendages (15 of 16 phages assessed) classified as species Ia, while a second species Ib is represented by a single isolate that did not display the globular appendages in its baseplate, but was shown to contain extended Y-shaped appendages (Ali et al., 2013). Phages capable of infecting *Ln. pseudomesenteroides* (group II) are grouped into four sub-groups and all present with a smaller baseplate structure than their *Ln. mesenteroides*-infecting counterparts (25 nm vs. 40 nm). Phages possessing a distinct collar structure below the phage head were classified as group IIa, while those without a collar were termed members of group IIb. A third group, IIc, is composed of isolates presenting with a "fluffy" baseplate appendage while the fourth group, IId, contains members that display unusual striations in the phage tail (Ali et al., 2013). In contrast to *Ln. mesenteroides* and *pseudomesenteroides*, phages infecting *Leuconostoc lactis* are rarely reported, representing a major knowledge gap in terms of the overall taxonomy of dairy *Leuconostoc* phages (Johansen and Kibenich, 1992), however, this underrepresentation may be due to the relatively low levels of usage of strains of this species in dairy

fermentations (Zamfir et al., 2006). The morphological diversity of phages infecting *Leuconostoc* species is quite striking given the limited number of strains that are available in the dairy setting. Considering the important role of *Leuconostoc* strains in flavor and aroma development in many fermented dairy products, this may represent an interesting and emerging area of LAB phage research. The isolation and characterization of further phages and of the dominant species as well as those of *Ln. lactis* would permit the development of further classification schemes and increasingly sophisticated detection tools for *Leuconostoc* phages, perhaps allowing a correlation to be made between phage prevalence and flavor development (or lack/reduction thereof), thus revealing the exact role of *Leuconostoc* strains within a given fermentation.

Lactobacillus PHAGES

Lactobacillus species are widely used as starter and adjunct cultures for certain food fermentations including the production of yoghurt, cheese, sauerkraut, pickles, and, in conjunction with yeasts, sourdough (Lu et al., 2003; Foschino et al., 2005). Some are used in the dairy industry for their purported probiotic effects (Felis and Dellaglio, 2007). In addition to these food fermentation uses of lactobacilli, some species are associated with food spoilage, e.g., *Lactobacillus casei* and *Lactobacillus brevis* are common beer spoilers (Asano et al., 2007). *Lactobacillus* phages belonging to the families *Siphoviridae* and*Myoviridae* have been isolated, while only a single *Lactobacillus* phage described thus far belongs to the *Podoviridae* family (Ackermann, 2007; Villion and Moineau, 2009). There is a relative paucity of genomic information regarding phages infecting members of this large and diverse genus, and there is limited taxonomic data regarding these phages (Mahony et al., 2012a). There are over distinct 100 species recognized within the *Lactobacillus* genus and with such host heterogeneity, it seems unsurprising that phages infecting species of this genus are equally complex and difficult to classify (Claesson et al., 2007). Currently, *Lactobacillus* phages are primarily classified based on the host species and subsequently into morphological or host range specific groups for a second tier of classification (For an extensive review of these phages, see Villion and Moineau, 2009). To date, the phage genomes of 24 *Lactobacillus* phages have been fully sequenced (http://www.ebi.ac.uk/genomes/phage.html) and their genetic complexity is clear with genome sizes ranging from ∼31–42 kb. It is possible that with increased genome sequence data, identification of taxonomic groups for this diverse genus may be possible. *Lactobacillus* phages also exhibit morphological diversity and this characteristic may thus be used in their differentiation and taxonomy.

CURRENT LIMITATIONS AND FUTURE PERSPECTIVES

Taxonomy of LAB-infecting phages has been the cornerstone of the development of detection and control tools, particularly pertaining to dairy fermentations. For example, several multiplex PCR systems have been established for the detection of lactococcal, S. *thermophiles*, and *Leuconostoc* phages (Labrie and Moineau, 2000; Del Rio et al., 2007, 2008; Ali et al., 2013). Such systems are essential to fermentation industries which rely on rapid identification of potentially problematic phages in order to limit phage proliferation within a plant. The practical relevance of phage taxonomy by far outweighs the apparent redundancy of repeated phage isolation, characterization and genomics studies as novel genetic elements, emerging phage species and evolving genome sequences continue to emerge. The vast information that currently exists for lactococcal phages has provided a solid basis for classification phages of LAB and other Gram positive bacteria. This data is based on more than three decades of isolation and characterization studies and genome sequencing efforts and have compounded the need for continual monitoring of phage populations. The loss of certain species (as single phage isolates may represent an entire species) and the identification of emerging and evolving phages present a significant challenge to phage taxonomy. With the exception of the Felix d'Herelle reference center for bacterial viruses in Canada, the general lack of centralized phage collection centers or the low uptake on requests for deposition of phage isolates in such collection centers is another issue that limits phage preservation and some phage isolates/species become obsolete if phage stocks are not maintained. Added to this is the lack of uniformity of classification methods. Classical studies relied upon serotyping and DNA-DNA hybridizations, which are time-consuming and not entirely discerning. In contrast, modern methodologies are becoming more reliant on genome sequencing, which has been possible through significant advances in sequencing technologies and throughput (Ronaghi et al., 1998; Eid et al., 2009; Meyer and Kircher, 2010). These advances together with the reduced cost of sequencing will be central to improving our knowledge of complex phage taxonomy groups, such as those represented by phages of the lactobacilli and those of underrepresented genera, including *Weisella*, *Oenococcus*, non-dairy *lactococci*, and *Leuconostoc* spp. It is evident that combinatorial strategies in phage taxonomy are still as useful today as they were in the past. Genomics combined with microscopic analysis is the current standard approach toward the classification of LAB phages with a decreased need for serotyping and exhaustive hybridization studies. One of the first attempts at unifying phage taxonomy was in 2002 by selecting a single structural protein (capsid or tail) as a phylogenetic marker and through this effort, *Siphoviridae* phages were classified into four groups (Proux et al., 2002). Following this, more sophisticated proteomic trees using overall proteomic data have been developed as a genome-based strategy for classifying phages. In 2002, the first phage proteomic tree of 105 phages was constructed. In this study, the *Siphoviridae* LAB-infecting phages clustered into one group of the proteomic tree, which may be sub-divided into the monophyletic taxonomic groups: sk1-like, TP901-1-like, Sfi21-like and the λ-like phages (Rohwer and Edwards, 2002). The *Siphoviridae* phages displayed most heterogeneity, while phages belonging to the remaining taxonomic groups (e.g., *Myoviridae* and *Podoviridae*) clustered into neat groups. This system places the LAB-infecting phages as part of the broader community of sequenced phages and such classification schemes are essential to understanding the overall relatedness and evolution of phages. In more recent years, this system has been expanded upon for *Myoviridae* and *Podoviridae* phages (Lavigne et al., 2006, 2009) and has endorsed the application of proteomics as a classification tool.

It is likely that LAB phage research will continue to focus on those phages that infect industrially significant genera as have been described above. It is also evident that the taxonomy of these phages requires regular review as the lessons learned from lactococcal phage taxonomy highlight the adaptive nature of phages in response to selective pressures in the industrial setting or the availability of alternative hosts (Fortier et al., 2006; Garneau et al., 2008). Therefore, phage taxonomy should be considered a fluid process that reflects the dynamic industrial environment which phages inhabit.

ACKNOWLEDGMENTS

Douwe van Sinderen is the recipient of a Science Foundation Ireland (SFI) Principal Investigator award (Ref. No. 08/IN.1/B1909).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 02 December 2013; paper pending published: 22 December 2013; accepted: 07 January 2014; published online: 24 January 2014.

*Citation: Mahony J and van Sinderen D (2014) Current taxonomy of phages infecting lactic acid bacteria. Front. Microbiol. **5**:7. doi: 10.3389/fmicb.2014.00007*

This article was submitted to Virology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 28 April 2014
doi: 10.3389/fmicb.2014.00186

Bacteriophages of *Leuconostoc*, *Oenococcus*, and *Weissella*

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Leuconostoc (*Ln.*), *Weissella*, and *Oenococcus* form a group of related genera of lactic acid bacteria, which once all shared the name *Leuconostoc*. They are associated with plants, fermented vegetable products, raw milk, dairy products, meat, and fish. Most of industrially relevant *Leuconostoc* strains can be classified as either *Ln. mesenteroides* or *Ln. pseudomesenteroides*. They are important flavor producers in dairy fermentations and they initiate nearly all vegetable fermentations. Therefore, bacteriophages attacking *Leuconostoc* strains may negatively influence the production process. Bacteriophages attacking *Leuconostoc* strains were first reported in 1946. Since then, the majority of described *Leuconostoc* phages was isolated from either dairy products or fermented vegetable products. Both lytic and temperate phages of *Leuconostoc* were reported. Most of *Leuconostoc* phages examined using electron microscopy belong to the *Siphoviridae* family and differ in morphological details. Hybridization and comparative genomic studies of *Leuconostoc* phages suggest that they can be divided into several groups, however overall diversity of *Leuconostoc* phages is much lower as compared to, e.g., lactococcal phages. Several fully sequenced genomes of *Leuconostoc* phages have been deposited in public databases. Lytic phages of *Leuconostoc* can be divided into two host species-specific groups with similarly organized genomes that shared very low nucleotide similarity. Phages of dairy *Leuconostoc* have rather limited host-ranges. The receptor binding proteins of two lytic *Ln. pseudomesenteroides* phages have been identified. Molecular tools for detection of dairy *Leuconostoc* phages have been developed. The rather limited data on phages of *Oenococcus* and *Weissella* show that (i) lysogeny seems to be abundant in *Oenococcus* strains, and (ii) several phages infecting *Weissella cibaria* are also able to productively infect strains of other *Weissella* species and even strains of the genus *Lactobacillus*.

Keywords: bacteriophages, *Leuconostoc*, *Oenococcus*, *Weissella*, morphogenesis, DNA sequence analysis

INTRODUCTION

TAXONOMY OF *Leuconostoc*, *Oenococcus*, AND *Weissella*

Leuconostoc (*Ln.*), *Weissella* (*W.*), *Oenococcus* (*O.*), and *Fructobacillus* (*F.*) form a group of related genera of lactic acid bacteria. Based on 16S rRNA sequencing, Collins et al. (1993) proposed that *Ln. paramesenteroides* and related species (*Lactobacillus (Lb.) confusus, Lb. halotolerans, Lb. kandleri, Lb. minor, and Lb. viridescens*) should be reclassified in the new genus *Weissella*. Dicks et al. (1995) assigned *Ln. oenos* to the new genus *Oenococcus*. Endo and Okada (2008) proposed to allocate several *Leuconostoc* species to the new genus *Fructobacillus*. Schleifer (2009), on the basis of 16S rRNA sequences, transferred the three genera *Leuconostoc* (including those species synonymous with *Fructobacillus*), *Weissella*, and *Oenococcus* into the newly formed family *Leuconostocaceae*. Members of the family show highest similarity to the genus *Lactobacillus*: they all are Gram-positive, catalase-negative, facultative anaerobes, and are characterized by heterofermentative lactic acid fermentation. While all members of the genera *Leuconostoc* and *Oenococcus* exhibit ovoid-shaped morphology, members of the genus *Fructobacillus* are rod-shaped. Species within the genus *Weissella* show two different (i.e., rod-shaped and ovoid-shaped) morphotypes.

SPECIES IN THE FAMILY *Leuconostocaceae*

According to information presented on the web-site of the "List of prokaryotic names with standing in nomenclature" (http://www.bacterio.net) the genus *Leuconostoc* is represented by 23 species and 4 subspecies for *Ln. mesenteroides*. However, several of the species names are synonyms within the genus *Leuconostoc* (like, e.g., *Ln. argentinum* and *Ln. lactis*, or *Ln. mesenteroides* subsp. *cremoris* and *Ln. cremoris*) or within different genera (like, e.g., *Ln. pseudoficulneus* and *F. pseudoficulneus*, *Ln. paramesenteroides* and *W. paramesenteroides*, *Ln. oenos* and *O. oeni*). Besides being recognized as meat spoilage organisms, *Leuconostoc* species have been described to be involved in several fermentation processes (Björkroth and Holzapfel, 2006). *Ln. mesenteroides* subsp. *mesenteroides*, *Ln. mesenteroides* subsp. *cremoris*, *Ln. lactis*, and *Ln. pseudomesenteroides* are regular constituents of aroma-producing

starter-cultures applied in dairy fermentations (Farrow et al., 1989). In addition, *Ln. mesenteroides* subsp. *mesenteroides* is an important component of vegetable fermentations: it is involved in fermentation of coffee beans and (together with *Ln. fallax*) in sauerkraut fermentation. *Ln. mesenteroides*, *Ln. citreum*, *Ln. gelidum* and *Ln. kimchii* are dominant species in early kimchi fermentation, *and Ln. mesenteroides* subsp. *dextranicum* plays a key role in sourdough fermentations (Schleifer, 2009).

The genus *Weissella* comprises 18 species. As already mentioned for the genus *Leuconostoc*, some of the species names are synonyms within the genus (like, e.g., *W. cibaria* and *W. kimchii*) or within different genera (like the above mentioned *Lactobacillus* species proposed to be reclassified as *Weissella*). The species *W. cibaria*, *W. confuse*, and *W. koreensis* have been described to be associated with vegetable fermentations (Schleifer, 2009). Recently, *W. fabalis* and *W. paramesenteroides* have been detected in cocoa bean fermentation (Snauwaert et al., 2013) and in traditional Caciocavallo cheese (Settanni et al., 2012), respectively.

The genus *Oenococcus* comprises just two species: *O. oeni* originally described as *Ln. oenos*, and *O. kitaharae* isolated from composting residues of schochu distillation (Endo and Okada, 2006). *O. oeni* plays an important role in wine fermentation, where it decarboxylates malic acid to lactic acid (Schleifer, 2009).

The genus *Fructobacillus* is represented by five species, all of which except *F. tropaeoli* are synonyms of *Leuconostoc* species (Endo et al., 2011). Species of this genus have been described to be involved in spontaneous cocoa bean fermentations (Snauwaert et al., 2013). So far, no bacteriophages have been described for this genus. Therefore, the fructobacilli will not be further addressed in this review.

HISTORY OF BACTERIOPHAGES IN THE FAMILY *Leuconostocaceae*

The first description of bacteriophages affecting *Leuconostoc* was published in 1946 (Mosimann and Ritter, 1946). In this publication already, the negative impact on butter aroma of bacteriophages infecting *Leuconostoc* strains was shown. Just 1 year later, Leiva-Quiros and McCleskey (1947) isolated phages infecting *Ln. mesenteroides* for phage-typing purposes. From late 1970's to beginning of 2000's only dairy *Leuconostoc* phages have been reported on with the exception of one report also included phages from coffee fermentations (**Table 1**) (Boizet et al., 1992). From 2002 to 2012 a number of reports on *Leuconostoc* phages from sauerkraut fermentations have been published, and since 2010–2012 genomes of *Ln. mesenteroides* and *Ln. pseudomesenteroides* phages have been published (**Table 1**). A thorough classification of dairy *Leuconostoc* phages has been presented recently (Ali et al., 2013).

Lu et al. (2003) reported on bacteriophages infecting *Weissella* sp. Later, several studies described *Podoviridae*-phages infecting *W. cibaria* (Pringsulaka et al., 2011; Kleppen et al., 2012b).

Sozzi et al. (1976) were the first to describe phage infecting lactic acid bacteria in wine, which were later identified as *O. oeni* (Sozzi et al., 1982; Dicks et al., 1995). Lysogeny appears to be rather frequent in *O. oeni*, with 45–60% of *O. oeni* strains reported to be lysogenic (Arendt et al., 1991; Poblet-Icart et al., 1998). Pan-genome comparisons have confirmed these results and have demonstrated that apparently six different bacterial tRNA genes are involved as targets for prophage DNA integration of temperate bacteriophages in different strains of *O. oeni* (Borneman et al., 2012).

FERMENTATIONS AFFECTED BY PHAGES INFECTING SPECIES OF *Leuconostocaceae*

Dairy fermentations are the most frequently described fermentations affected by bacteriophages (Samson and Moineau, 2013). This may be due to two major reasons: (i) milk is a liquid substrate in which phage are easily distributed, and (ii) most dairy fermentations involve application of starter culture mixtures, thus variations in acidification performance become readily evident. Presence of phages infecting dairy *Leuconostoc* strains has only been described occasionally (Sozzi et al., 1978; Boizet et al., 1992; Davey et al., 1995). However, the publications never acquired attention similar to those describing phages causing disturbances of acidification. This is probably due to the fact that during fermentation acidification failures are much easier and much earlier detectable than aroma defects. As a consequence, while the negative impact of phage on starter strains and acidification is well documented, the impact of phage on starter strains and aroma development is much less well known and is only beginning to be investigated systematically (Samtlebe et al., 2012). In recent years Swedish and Danish dairies have reported problems related to lack of diacetyl and CO_2 in fermented milks (similar to report by Mosimann and Ritter, 1946) that could be correlated to phages attacking *Leuconostoc* strains (Anon. meeting reports, Vogensen, not published). Similarly, in several cases phage attacks on *Leuconostoc* strains in blue-mold cheeses have been correlated with lack of mold growth probably due to less openness in the cheese structure (Kot et al., 2014; Pujato et al., 2014).

Highest *Leuconostoc* phage titers in dairy products or in whey samples can vary significantly within a range from approximately 10^2 to 10^7 plaque-forming units (PFUs) per gram or per ml (Atamer et al., 2011) (**Figure 1**). The maximum numbers of PFUs for *Leuconostoc* phages in dairy samples are approximately 2 log units lower than maximum lactococcal phage numbers (approximately 10^9 PFU/ml). The lower *Leuconostoc* phage numbers are therefore probably reflecting the use of *Leuconostoc* as a minor starter component (1–10%) in undefined complex cultures consisting mainly of lactococcal strains. While the homo-lactic lactococci mainly contribute to acidification, the heterolactic and only weakly acidifying leuconostocs contribute to aroma by production of acetate, acetoin, and diacetyl (Farkye and Vedamuthu, 2002).

The *Leuconostoc* lytic phages involved in dairy fermentations have generally been shown to be members of the *Siphoviridae* group of phages (Davey et al., 1995; Kleppen et al., 2012a; Ali et al., 2013; Kot et al., 2014; Pujato et al., 2014). However, for *Leuconostoc* phages isolated from sauerkraut fermentations also *Myoviridae* phages are seen (Barrangou et al., 2002; Yoon et al., 2002; Lu et al., 2003). Other than in dairy fermentations, in sauerkraut fermentations phages may even play an important role by affecting the development of different lactic acid bacteria species over fermentation time (Lu et al., 2003). However, when defined starter strains are supposed to be applied, phage infection may negatively affect quality parameters of the final

Table 1 | Table summarizing reports on phages infecting genus *Leuconostoc*.

***Leuconostoc* host species**	**Origin**	**Life style**	**Analysis**	**References**
Ln. citrovorum (*Ln. mesenteroides* subsp. *cremoris*)	Dairy	Lytic	Flavor defects	Mosimann and Ritter, 1946
Ln. mesenteroides	Dairy	Lytic	TEM (2 phages)	Sozzi et al., 1978
Ln. mesenteroides subsp. *cremoris*, subsp *dextranicum*, and subsp. *mesenteroides*	Dairy	Lytic (4 phages)	Host range	Shin and Sato, 1979
Ln. mesenteroides subsp. *dextranicum* and subsp. *mesenteroides*	Dairy	Temperate	TEM (9 phages)	Shin and Sato, 1979
Ln. mesenteroides subsp. *cremoris*	Dairy	Lytic (phage Lc-4)	1-step growth	Shin, 1983
Ln. mesenteroides subsp. *cremoris*	Dairy	Lytic (4 phages)	Host range, TEM	Saxelin et al., 1986
Ln. mesenteroides subsp. *cremoris*	Dairy	Lytic (phage PWL-2)	TEM, structural proteins, REN analysis	Neve et al., 1988
Ln. mesenteroides subsp. *cremoris* and subsp. *Ln. lactis*	Dairy	Lytic (4 phages)	Host range	Johansen and Kibenich, 1992
Ln. mesenteroides	Dairy, coffee	Lytic (19 phages)	6 DNA homology groups, structural proteins, TEM, REN analysis, genome sizes	Boizet et al., 1992
Leuconostoc sp.	Dairy	Lytic (4 phages)	1 DNA homology group, structural proteins, TEM, REN analysis	Davey et al., 1995
Ln. mesenteroides and *Leuconostoc sp.*	Sauerkraut	Lytic (8 phages)	TEM, Host range	Yoon et al., 2002
Ln. fallax	Sauerkraut	Lytic (6 phages)	TEM, REN analysis, Host range, structural proteins	Barrangou et al., 2002
Ln. mesenteroides, Ln. citreum, Ln. pseudomesenteroides, Ln. fallax	Sauerkraut	Lytic (29 phages)	Host range (all), TEM, REN analysis, structural proteins (6 phages)	Lu et al., 2003
Ln. mesenteroides	Sauerkraut	Lytic (Φ1-A4)	TEM, structural proteins, genome sequence (29.5 kb)	Lu et al., 2010
Ln. pseudomesenteroides	KC04 strain	Temperate (ΦMH1)	TEM, genome sequence (38.7 kb)	Jang et al., 2010
Ln. mesenteroides; Ln. pseudomesenteroides	Dairy	Lytic (77 phages)	Host range, thermal stability and inactivation kinetics, TEM	Atamer et al., 2011
Ln. mesenteroides (pseudomesenteroides)	Dairy	Lytic (phage Lmd1)	TEM, genome sequence (26.2 kb)	Kleppen et al., 2012a
Ln. mesenteroides and *pseudomesenteroides*	Dairy	Lytic (83 phages)	TEM, 2 DNA homology groups, host range, PCR detection	Ali et al., 2013
Ln. pseudomesenteroides	Dairy	Lytic (2 phages)	TEM, receptor binding proteins	Kot et al., 2013
Ln. mesenteroides and *Ln. pseudomesenteroides*	Dairy	Lytic (9 phages)	TEM, genome sequence, structural proteins, host range	Kot et al., 2014
Ln. mesenteroides subsp. *mesenteroides*	Dairy	Lytic (9 phages)	TEM, stability and inactivation kinetics, REN analysis, host range	Pujato et al., 2014

Type of analysis presented in the paper is listed in the column "analysis." TEM, Transmission electron microscopy, REN, analysis of restriction endonuclease fragments.

product (Mudgal et al., 2006). Applying metagenomic analysis to kimchi, a traditional Korean fermented cabbage, evidence for presence of phage infecting *Leuconostoc* was obtained (Jung et al., 2011). So far, the only phage/host pair characterized for kimchi is a *Podoviridae* phage infecting *Weissella cibaria* (Kleppen et al., 2012b). A similar pair, *Podoviridae* phage and W. *cibaria* host, has been described for Nham, a Thai fermented pork sausage (Pringsulaka et al., 2011). Recently, several phages infecting *W. cibaria* and *W. paramesenteroides* were isolated from commercial cucumber fermentations and one phage for each host was shown to belong to the *Siphoviridae* family of phages (Lu et al., 2012). The only fermentation known to be affected by phages infecting *O. oeni* is wine fermentation, due to the exclusive involvement of these host bacteria in this type of fermentation (Schleifer, 2009). The phages have been shown to belong to the *Siphoviridae* family of phages (Poblet-Icart et al., 1998).

Leuconostoc PHAGES

Phages attacking *Leuconostoc* are best documented among all phages of the *Leuconostocaceae* family. The majority of reports on *Leuconostoc* phages are connected to problems in dairy fermentations, however few of the reports are dealing with *Leuconostoc* phages in vegetable or in coffee fermentations (**Table 1**). However, *Leuconostoc gelidum* is a known meat spoilage organism (Sakala et al., 2002). Interestingly, it was proposed to use *Ln. gelidum* phages to prevent bacterial spoilage of the meat products, pointing toward a different angle of phage-host interactions, i.e., phage bioprotection in fermented foods (Greer et al., 2007).

MORPHOLOGY OF *Leuconostoc* PHAGES

Recently, the morphotypes of dairy *Ln. pseudomesenteroides* and of *Ln. mesenteroides* phages from the dairy environment have been studied extensively with a set of 83 phage isolates (Ali et al. (2013). Although the phages were isolated from various sources (11 dairies, 3 phage collections), a low degree of variation was documented for their morphotypes. All phages were small isometric-headed *Siphoviridae* phages with non-contractile 140-nm long tails, however, according to their baseplate structure, these phages were differentiated into 6 different subgroups with six globular baseplate appendices or with peculiar Y-shaped baseplate structures (*Ln. mesenteroides* phages of morphotypes Ia or Ib), with plain baseplates but with or without characteristic collar structures or with uncommon tail striations (*Ln. pseudomesenteroides* phages of morphotypes IIa, b and d), or with undefined "fluffy" baseplate appendices (morphotype IIc *Ln. pseudomesenteroides* phages) (**Figure 2**). Dairy *Leuconostoc* phages of morphotypes Ia and IIb had been reported occasionally, i.e., Ia type phages: (Neve et al., 1988); IIb type phages: (Saxelin et al., 1986; Davey et al., 1995; Kleppen et al., 2012a). *Siphoviridae* phages of *Leuconostoc* with longer phage tails have also been described previously, indicating a broader biodiversity (Saxelin et al., 1986) within *Leuconostoc* phage populations. This correlates well with the establishment of 6 DNA homology groups for *Ln. mesenteroides* phages (Boizet et al., 1992). *Leuconostoc* phages isolated from sauerkraut fermentations did also reveal different morphotypes *Siphoviridae* phages with different tail lengths and *Myoviridae* phages (Barrangou et al., 2002; Lu et al., 2003, 2012). Temperate *Siphoviridae* phages from lysogenic *Ln. mesenteroides* and *Ln. pseudomesenteroides* strains with different tail lengths have been shown by Shin and Sato (1979), Lu et al. (2012), and Jang et al. (2010).

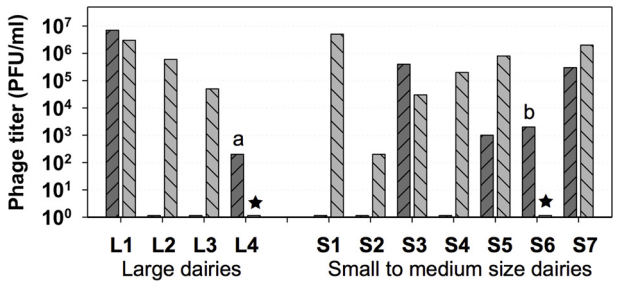

FIGURE 1 | Highest *Leuconostoc* phage titers in whey ( ) and in brine ( ) samples obtained from 4 large and from 7 small to medium size dairies. ★, no brine samples (dairies L4 and S6); a, butter milk sample; b, butter cream sample. (Modified from Atamer et al., 2011).

GENETICS OF *Leuconostoc* PHAGES

Currently, there are 12 full genomes of phages infecting *Leuconostoc* sp. present in publically available databases. All phages have dsDNA genomes with sizes from 25.7 to 38.7 kb (**Table 2**). Genomic G + C content varies from 36.1% for phage Φ-A4 to 38.7% for phage ΦMH1. All described lytic phages of *Leuconostoc* exhibit high similarity in regard to genome organization. Five modules can be distinguished in the genomes specifying replication, packaging, morphogenesis, host cell lysis, and regulation and modification. Moreover, high similarity of putative proteins encoded in the genomes of lytic *Leuconostoc* phages suggests that they originated from a common ancestor (Kot et al., 2014). Ali et al. (2013) reported two groups of hybridization patterns among lytic *Leuconostoc* phages of dairy origin; one for *Ln. mesenteroides* phages and one for *Ln. pseudomesenteroides* phages, although all phage members of the two groups share a short, cross-hybridizing genome region. The cross-hybridizing region codes for tail proteins, e.g., major tail protein (*mtp*) and tape measure protein (*tmp*), however higher similarities were found within *mtp* gene. This conserved region was used as target for developing a universal PCR-based detection system for lytic phages of *Ln. mesenteroides* and *Ln. pseudomesenteroides* (Ali et al., 2013). The PCR assay resulted in 322-bp long fragments and was validated with all reported 83 lytic phages of *Leuconostoc* (Ali et al., 2013). The recent sequencing data confirms that the selected region is

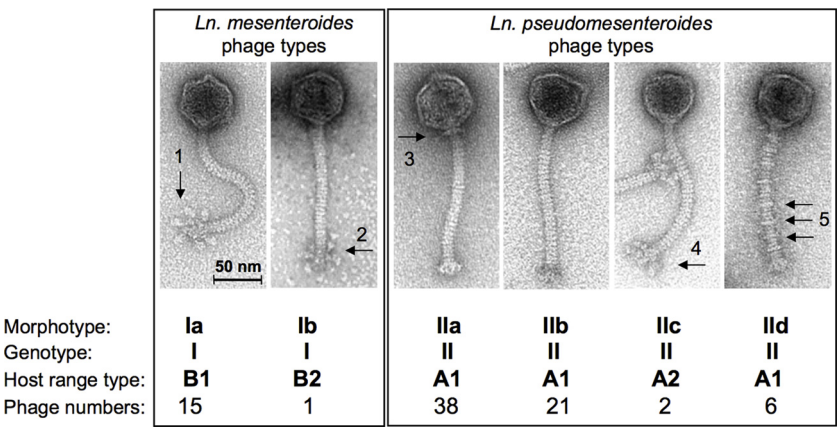

FIGURE 2 | Overview on the current taxonomy of dairy *Leuconostoc* phages based on transmission electron microscopy, genotyping, and host range profiles. Arrows indicate structural details as follows: globular baseplate appendices (1), non-globular (Y-shaped) baseplate appendages (2), collar or neck passage structure (3), "fluffy" baseplate appendices (4), tail striations (5). (modified from Ali et al., 2013).

indeed the only region that can be used for PCR-based detection for both phage species (Kot et al., 2014).

Genomes of lytic phages of *Leuconostoc* contain from 38 to 50 predicted genes. Some of us were involved in biological characterization of one of the genes present in *Ln. pseudomesenteroides* phages, namely the receptor binding protein (RBP) (Kot et al., 2013). Construction of chimeric phages resulted in the transition in host range allowing the identification of the receptor binding protein genes to be ORF21$_{P793}$ and ORF23$_{\Phi LN04}$, respectively. Until now, the host-encoded receptor for *Leuconostoc* phages remains unknown.

Currently, there is only one complete genome sequence of a temperate phage attacking *Leuconostoc* deposited in public databases. The phage is designated ΦMH1 and it was obtained from a UV-induced lysate of *Ln. pseudomesenteroides* strain KC04 (Jang et al., 2010). No host for ΦMH1 phage was reported. ΦMH1 has a dsDNA genome with a length of 38.7 kb with 65 putative ORFs identified. ΦMH1 did not show significant similarities with other described phages of *Leuconostoc* (Jang et al., 2010). Besides of ΦMH1 phages, several predicted prophages can be identified in the sequenced genomes of *Leuconostoc* (**Table 3**). Analysis of complete translatome of fully sequenced phages and prophages shows that diversity of prophage elements is higher than within sequenced two groups of lytic phages (**Figure 3**).

Oenococcus PHAGES

Phages attacking *O. oeni* were reported already in the late 1960's and beginning of 1970's (Sozzi et al., 1976), and 3 phage

Table 2 | Bacteriophages of *Leuconostoc* and *Weissella* with complete genome sequences deposited in public databases.

Genus	Name	Host[a]	Accession nr	Information	Genome size (kb)	References
Leuconostoc	Φ1-A4	*Ln. mesenteroides* 1-A4	GQ451696	Lytic, *cos*-type	29.5	Lu et al., 2010
Leuconostoc	ΦLmd1	*Ln. mesenteroides* ssp. *dextranicum* A1	NC_018273	Lytic, *cos*-type	26.2	Kleppen et al., 2012a
Leuconostoc	ΦLN25	*Ln. mesenteroides* LN25	KC013026	Lytic, *cos*-type	28.4	Kot et al., 2014
Leuconostoc	ΦLN34	*Ln. mesenteroides* LN05	KC013027	Lytic, *cos*-type	28.0	Kot et al., 2014
Leuconostoc	ΦLNTR2	*Ln. mesenteroides* LN05	KC013028	Lytic, *cos*-type	28.3	Kot et al., 2014
Leuconostoc	ΦLNTR3	*Ln. mesenteroides* LN05	KC013029	Lytic, *cos*-type	28.0	Kot et al., 2014
Leuconostoc	P793	*Ln. pseudomesenteroides* BM2	NC_020880	Lytic, *cos*-type	26.8	Kot et al., 2013
Leuconostoc	ΦLN04	*Ln. pseudomesenteroides* LN02	NC_020870	Lytic, *cos*-type	25.9	Kot et al., 2013
Leuconostoc	ΦLN03	*Ln. pseudomesenteroides* LN02	KC013022	Lytic, *cos*-type	26.8	Kot et al., 2014
Leuconostoc	ΦLN12	*Ln. pseudomesenteroides* LN02	KC013025	Lytic, *cos*-type	28.2	Kot et al., 2014
Leuconostoc	ΦLN6B	*Ln. pseudomesenteroides* LN02	KC013024	Lytic, *cos*-type	25.7	Kot et al., 2014
Leuconostoc	ΦMH1	NA[a]	HM596271	Induced from *Ln. pseudomesenteroides* KC04	38.7	Jang et al., 2010
Weissella	ΦYS61	*Weissella cibaria* YS61	NC_018270	Lytic, protein dependent DNA packaging	33.6	Kleppen et al., 2012b

Table 3 | Predicted prophage sequences found in fully assembled chromosomes of *Leuconostoc, Oenococcus,* and *Weissella* available in GenBank.

Species	Strain	Accession nr	Number of predicted prophages	Size of predicted prophages (kb)
Leuconostoc mesenteroides	ATCC 8293	NC_008531	1	41.9
Leuconostoc mesenteroides	J18	NC_016805	0	
Leuconostoc citreum	KM20	NC_010471	1	50.5
Leuconostoc gelidum	JB7	NC_018631	0	
Leuconostoc gasicomitatum	LMG 18811	NC_014319	2	11.5, 45.1
Leuconostoc sp.	C2	NC_015734	1	37.5
Leuconostoc carnosum	JB16	NC_018673	0	
Leuconostoc kimchi	IMSNU 11154	NC_014136	3	13.1, 36.8, 65
Oenococcus oeni	PSU-1	NC_008528	0	
Oenococcus kitaharae	DSM 17330	NZ_CM001398	0	
Weissella koreensis	KACC 15510	NC_015759	0	

Prediction was done using PHAST (Zhou et al., 2011) and PhiSpy (Aziz et al., 2008; Akhter et al., 2012) and manually verified afterwards.

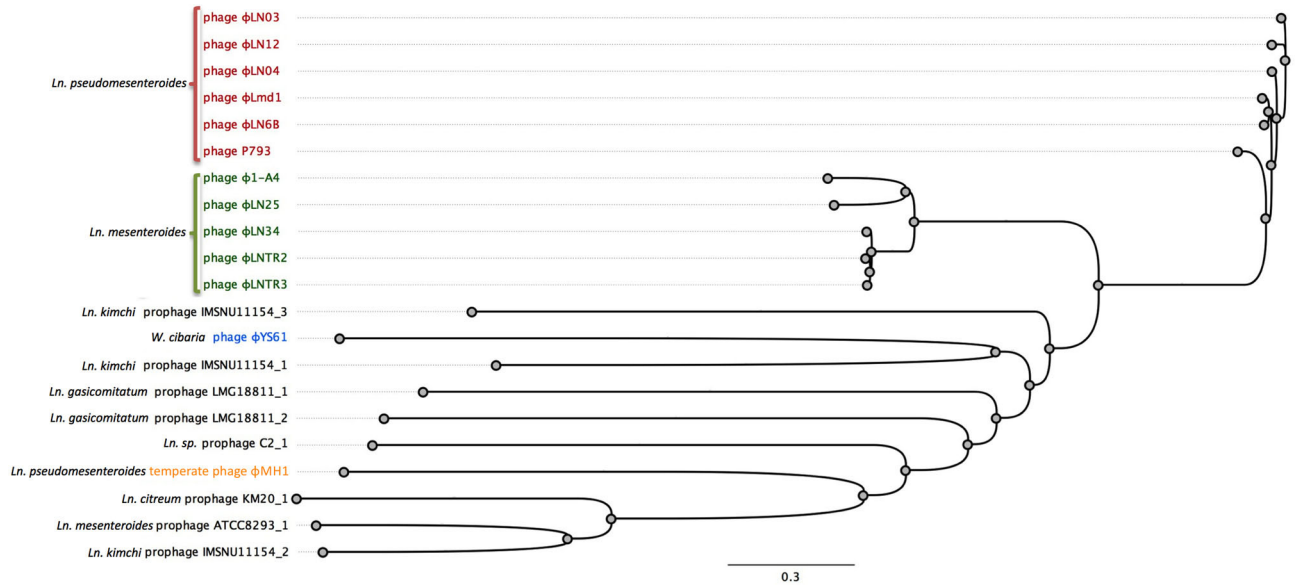

FIGURE 3 | Comparison of the complete translatome of fully sequenced phages and predicted prophages from complete genomes of *Leuconostoc, Weissella,* and *Oenococcus*. Genomes were prepared as described before (Mahony et al., 2013). The concatemeric sequences were aligned using Geneious 7.1.2 software using BLOSUM62 cost matrix. The phylogenetic tree was constructed and visualized with bootstrapped neighbor-joining method with 500 replicates in Geneious 7.1.2 software.

morphotypes were described for phages isolated from wine. They all had isometric heads and 3 distinct non-contractile tail lengths, i.e., belonging to *Siphoviridae*. Later, lytic phages from four Australian wine areas attacking approximately 40% of *O. oeni* isolates from the same regions were isolated (Davis et al., 1985). These also were belonging to *Siphoviridae* with isometric heads and long non-contractile tails (approximately 300 nm). Subsequently, phage P58I was isolated from a phage carrying culture of *O. oeni* 58N (Arendt et al., 1990). This phage was able to plaque on strain 58N as well as on 58PF, which was a phage-free derivative of 58N strain. A similar phage P58II isolated after mitomycin C induction of the 58N strain was not able to plaque on any of the two strains. Surprisingly no DNA homology was detected between the two phages genomes and the chromosome of *O. oeni* 58N.

Huang et al. isolated a temperate phage Φ1002 that was able to grow lytically on approximately 46% of all *O. oeni* isolates from Australian wine (Huang et al., 1996). The phage belonged to the *Siphoviridae* family with a 52 nm isometric head and a 210 nm non-contractile tail. A set of 17 prophages were induced from *O. oeni* isolated from Portuguese wines (Santos et al., 1998). They all had a similar morphology with isometric heads of approximately 40–50 nm and non-contractile tails of approximately 220–240 nm. The *cos*-type phages were divided into 6 groups based on restriction enzyme digestion profiles. These could further be divided into 2 main groups α and β based on restriction maps (Santos et al., 1998). Cross-hybridization between the α and β group was located in the central part of the genomes and included the phage attachment site (*attP*). This part was later sequenced (Parreira et al., 1999) and revealed the presence of lysin and holin genes.

The lysin (Lys44) from *O. oeni* phage fOg44 was described in greater detail. Interestingly, secretion of the lysin seems to occur with the aid of a signal peptide and independent of the holin, also encoded in the phage genome. A potential role of the holin as a triggering factor for lytic activity is discussed (São-José et al., 2000).

Screening of 167 isolates of *O. oeni* for lysogeny by mitomycin C resulted in the identification of approximately 45% of lysogenic strains and for some of these propagating hosts were identified as indicator strains (Poblet-Icart et al., 1998).

Until now, there is no complete genome sequence of a phage attacking *O. oeni*, however a number of partial sequences derived from phages of *O. oeni* have been deposited in public databases. Borneman et al. (2012) reported several prophage sequences in the *O. oeni* pan genome. Prophage-like sequences were integrated into six different tRNA genes, with some of these sequences representing presumably functional phages (Borneman et al., 2012). Recently, Doria et al. (2013) communicated a PCR-based method for detection and identification of lysogenic strains of *O. oeni*. The assay allowed detection of a target sequence within the prophage lysin gene in 25 out of 37 isolates tested. Furthermore, the majority of the lysogenic isolates could be prophage induced (Doria et al., 2013). Shortly after, Jaomanjaka et al. (2013) analyzed oenococcal prophages based on integrase gene polymorphism and classified them into four groups (A–D). Remarkably, in the two fully assembled chromosomes of *Oenococcus* sp. no prophage sequences were detected using PHAST and PhiSpy program (Aziz et al., 2008; Zhou et al., 2011; Akhter et al., 2012) (**Table 3**). Absence of prophage-like sequences in the *O. oeni* PSU-1 strain had been reported before by Mills et al. (2005).

Weissella PHAGES

Pringsulaka et al. (2011) isolated phage Φ22 attacking *Weissella cibaria* N22 from a Thai fermented pork sausage Nham. This phage belonged to the *Podoviridae* family with morphotype C2 with a prolate head of approximately 92 × 50 nm and a non-contractile tail of 37 nm. Phage Φ22 had a narrow host-range attacking only one of 40 *W. cibaria* strains.

Lu et al. (2012) also isolated phages attacking *W. cibaria* from the initial phase of cucumber fermentation. Interestingly, the host range of some of these phages crossed the species barrier and in some cases also the genus barrier. Phage Φ3.8.18 belonging to the *Myoviridae* family attacked two isolates of *W. cibaria,* one isolate of *Lb. plantarum* and one isolate of *Lb. brevis*. Phage Φ3.8.18 had an isometric head of approximately 80 nm and an approximately 200 nm tail with indication of a baseplate structure. Another *Myoviridae* phage Φ7.2.50 attacked same two isolates of *W. cibaria* and 24 isolates of *Lb. brevis*. Also two *Siphoviridae* phages crossed the species/genus barrier. Phage Φ3.8.43 attacked, beside four *W. cibaria* isolates, one isolate of *Lb. plantarum*, and one isolate of *Lb. brevis* (both of which were also attacked by Φ3.8.18). Phage Φ3.8.43 had an isometric head of approximately 50–60 nm and an approximately 250 nm long non-contractile tail. Besides two isolates of *W. cibaria,* phage Φ3.8.48 also attacked one isolate of *W. paramesenteroides*. Kleppen et al. (2012b) determined the genome sequence of ΦYS61 attacking *W. cibaria* (**Table 2**). This phage isolated from 1-week old kimchi fermentation belonged to the *Podoviridae* family of morphotype C2 (Ackermann, 1998) with a prolate head of 85 × 36 nm and a short non-contractile tail.

Phage ΦYS61 is infecting *W. cibaria* (Kleppen et al., 2012b) and has a 33.6 kb dsDNA genome, which is similar to the estimated genome size of another podovirus of *W. cibaria* isolated recently from a Thai sausage (Pringsulaka et al., 2011). The genome of the ΦYS61 phage codes for 48 putative ORFs. It is very likely that ΦYS61 utilizes a protein-dependent DNA replication mechanism similarly to Φ29 phage from *Bacillus subtilis* (Kleppen et al., 2012b). Very few putative genes of ΦYS61 show significant similarities to the sequences present in public databases. No prophages were detected in *W. koreensis* KACC 15510 strain (**Table 3**).

CONCLUSION

Phages of *Leuconostoc*, *Oenococcus*, and *Weissella* are present in many types of food-related fermentations, where they are responsible for various defects in production. The majority of described phages were isolated from dairy samples, where they attack *Leuconostoc* starter strains and subsequently contribute to aroma- and CO_2-production defects. Another large reservoir of *Leuconostoc* and *Weissella* phages are various vegetable fermentations, most importantly kimchi and sauerkraut fermentations. All phages of *Oenococcus* described so far are solely reported in connection to wine production, where they can disturb the malolactic fermentation.

All phages of *Leuconostoc*, *Oenococcus*, and *Weissella* belong to *Caudovirales* order with members of the *Siphoviridae*, *Podoviridae*, and *Myoviridae* families. Thirteen complete genomes of phages infecting *Leuconostoc* and *Weissella* have been reported. Among them, lytic phages of *Leuconostoc* belonging to *Siphoviridae* exhibit high similarities in overall composition, regardless on the environment they were isolated from. PCR-based assays for detecting lytic *Leuconostoc* and *Oenococcus* phages have been established so far, however further detailed knowledge of the genetic diversity of *Leuconostoc*, *Oenococcus*, and *Weissella* phages, e.g., *Myoviridae* phages from sauerkraut fermentations as well as temperate phages is needed in order to provide better taxonomy, control, and detection strategies for these groups of phages.

ACKNOWLEDGMENTS

Witold Kot was the recipient of a Ph.D. grant from University of Copenhagen (UoC). The work on dairy *Leuconostoc* phages done in our own labs at UoC and at Max Rubner-Institut (MRI) was partially financed by MetaPhageLAB (UoC: FTP project no. 11-106991) and was supported by a grant from the Forschungskreis der Ernährungsindustrie e.V. (Bonn, Germany), the AiF, and the Ministry of Economics and Technology (MRI: project no. AiF-FV 15886N). MRI authors sincerely acknowledge the cooperation with University of Hohenheim (J. Hinrichs and Z. Atamer) within project AiF-FV 15886N.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 27 December 2013; accepted: 04 April 2014; published online: 28 April 2014. Citation: Kot W, Neve H, Heller KJ and Vogensen FK (2014) Bacteriophages of Leuconostoc, Oenococcus, and Weissella. Front. Microbiol. ***5****:186. doi: 10.3389/fmicb.2014.00186*

This article was submitted to Virology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 13 January 2014
doi: 10.3389/fmicb.2013.00417

Phages of non-dairy lactococci: isolation and characterization of ΦL47, a phage infecting the grass isolate *Lactococcus lactis* ssp. *cremoris* DPC6860

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Lactococci isolated from non-dairy sources have been found to possess enhanced metabolic activity when compared to dairy strains. These capabilities may be harnessed through the use of these strains as starter or adjunct cultures to produce more diverse flavor profiles in cheese and other dairy products. To understand the interactions between these organisms and the phages that infect them, a number of phages were isolated against lactococcal strains of non-dairy origin. One such phage, ΦL47, was isolated from a sewage sample using the grass isolate *L. lactis* ssp. *cremoris* DPC6860 as a host. Visualization of phage virions by transmission electron microscopy established that this phage belongs to the family *Siphoviridae* and possesses a long tail fiber, previously unseen in dairy lactococcal phages. Determination of the lytic spectrum revealed a broader than expected host range, with ΦL47 capable of infecting 4 industrial dairy strains, including ML8, HP and 310, and 3 additional non-dairy isolates. Whole genome sequencing of ΦL47 revealed a dsDNA genome of 128, 546 bp, making it the largest sequenced lactococcal phage to date. In total, 190 open reading frames (ORFs) were identified, and comparative analysis revealed that the predicted products of 117 of these ORFs shared greater than 50% amino acid identity with those of *L. lactis* phage Φ949, a phage isolated from cheese whey. Despite their different ecological niches, the genomic content and organization of ΦL47 and Φ949 are quite similar, with both containing 4 gene clusters oriented in different transcriptional directions. Other features that distinguish ΦL47 from Φ949 and other lactococcal phages, in addition to the presence of the tail fiber and the genome length, include a low GC content (32.5%) and a high number of predicted tRNA genes (8). Comparative genome analysis supports the conclusion that ΦL47 is a new member of the 949 lactococcal phage group which currently includes the dairy Φ949.

Keywords: ***Lactococcus lactis*, non-dairy, phage, tail fiber, genome**

INTRODUCTION

Cultures of lactic acid bacteria (LAB) used in cheese manufacture play a pivotal role in the formation of cheese flavor (Urbach, 1995). While the limited numbers of established dairy cultures in use have greatly reduced inconsistencies in cheese quality, it can be at the expense of cheese flavor. LAB isolated from non-dairy environments, such as plant material, often exhibit enhanced metabolic capabilities when compared to those of dairy origin and have been shown to contribute to a more diverse flavor profile in the cheese (Ayad et al., 1999, 2000; Morales et al., 2003). In addition to their role in flavor enhancement, cultures of non-dairy origin have also been shown to be naturally insensitive to bacteriophages which infect industrial strains (Ayad et al., 2000). Phage infection is the single largest cause of industrial milk fermentation problems, negatively impacting on the consistency of cheese quality and resulting in large economic losses (Coffey and Ross, 2002). Non-dairy lactococcal strains could potentially be exploited for use in cheese culture rotations as phage insensitive strains and may well reduce the negative consequences of phage infection within a production plant. However, given the high evolution rate of phages, it is highly likely that over time, phages would emerge to threaten these bacterial strains.

All lactococcal phages isolated to date are members of the *Caudovirales* order which comprises of three families: *Siphoviridae* (long, non-contractile tails), *Myoviridae* (long, contractile tails) and *Podoviridae* (short tails) [for review see Veesler and Cambillau (2011)]. Phage classification systems have been utilized in the design of anti-phage strategies to prevent phages amplifying to high titers in manufacturing plants (Jarvis et al., 1991). DNA hybridization assays and comparative genomic analysis coupled with visualization of virions has led to the division of lactococcal phages into one of ten groups (Deveau et al., 2006). Of these phage groups, the c2, 936 and P335 groups are predominantly associated with failed dairy fermentations and are

frequently isolated from cheese manufacturing plants (Moineau et al., 1992; Murphy et al., 2013). Currently within the NCBI database, there are complete genome sequences for over 30 lactococcal bacteriophages. Phages classified within the same group can share a large degree of genome similarity as observed within the c2 and 936 groups (Lubbers et al., 1995; Chopin et al., 2001; Crutz-Le Coq et al., 2002). However, within the P335 group, there exists a high proportion of genetic diversity attributed to the presence of both temperate and virulent bacteriophages classified within this group (Labrie and Moineau, 2007; Garneau et al., 2008). Collectively, these groups account for the majority of sequenced lactococcal phage genomes, ranging in size from 22 to 40 kb. Larger lactococcal phages do also exist, with Φ949 at 114.8 kb being the largest sequenced lactococcal phage to date (Samson and Moineau, 2010). With the elucidation of the Φ949 genome, a genomic sequence is available for at least one member of each phage group.

The use of a wide range of different bacterial strains possessing an industrially important phenotype has presumably contributed to the diversity of lactococcal phages. Genome analysis of the rarer uncommon lactococcal phages has shed further light on this diversity. Comparative analysis of the structural proteins of Φ1358, show a high degree of similarity to 2 *Listeria monocytogenes* phages. Moreover this phage was also found to possess an uncommonly high %GC content of 51 (Dupuis and Moineau, 2010). Recent analysis of the Φ1706 genome, revealed that 22 ORFs shared similarities with proteins of Firmicutes, typically found in the human gut (Garneau et al., 2008). The ability of this phage to infect lactococci was attributed to the acquisition of a 4 gene module, allowing for host recognition of lactococcal cells (Garneau et al., 2008). Genomic analysis of the ΦKSY1 genome has suggested the exchange of genetic material between bacteria and phages from different environments (Chopin et al., 2007). ΦQ54 was found to possess a different modular configuration, thought to be derived from recombination events with 936 and c2 type phages (Fortier et al., 2006). It is probable that these rarer phages arose from recombination events with other lactococcal phages and phages infecting other Gram-positive bacteria (Dupuis and Moineau, 2010). Moreover, these phages appear to be less suited to thrive in milk fermentations in contrast to phages of the more common lactococcal phage groups which possess rapid reproduction rates believed to be driven by evolutionary pressure (Ferguson and Coombs, 2000; Dupuis and Moineau, 2010).

In this study we describe the isolation and characterization of ΦL47, a large lytic phage which infects the non-dairy isolate *L. lactis* ssp. *cremoris* DPC6860. We also report the complete genome sequence of ΦL47 which, to our knowledge, is the largest lactococcal phage reported to date. Due to the emergence of non-dairy lactococci as dairy cultures with enhanced flavor-forming activity, sufficient data needs to be generated with regards to these cultures and their phage resistance if they are to be successfully utilized in dairy processing. Therefore, the objective of this study was to provide a better understanding of phages of lactococci from non-dairy origins at a phenotypic and genomic level, thus offering further insight into phage-host interactions.

MATERIALS AND METHODS

BACTERIAL STRAINS, BACTERIOPHAGE AND CULTURE CONDITIONS

Dairy and non-dairy *Lactococcus* strains were supplied by the TFRC-Moorepark culture collection (Teagasc Food Research Centre, Moorepark, Ireland). *L. lactis* ssp. *cremoris* DPC6860 was previously isolated from grass (D. Cavanagh, unpublished) and characterized by 16S rRNA analysis as described by Alander et al. (1999). All lactococcal strains used in this study were cultured in M17 (Oxoid, Hampshire, England) media containing 0.5% lactose (wt./vol.) (VWR, Leuven, Belgium) (LM17) at 30°C for 18–24 h under aerobic conditions. Double-strength M17 broth, used for phage enrichment of sewage samples, was prepared by doubling the amount of dry M17 media and reconstituting it in the same amount of distilled water as the 1X media with the addition of 1% lactose (wt./vol.). Soft agar overlays and solid agar medium contained 0.75% and 1.5% agar, respectively. Lactococcal phages ΦKSY1, Φc2, ΦbIL170 and ΦP008 were originally obtained from the Felix d'Hérelle Reference Center for Bacterial Viruses (GREB, Pavillon de Médecine Dentaire Université Laval, QC, Canada) while phages Φ712, ΦHP, ΦebI and ΦML3 were obtained from the UCC culture collection (University College Cork, Cork, Ireland). Phages were propagated using their respective hosts at 30°C in M17 media containing 0.5% glucose (wt./vol.) (Sigma-Aldrich, Dublin, Ireland) and 10 mM $CaCl_2$.

SPOT ASSAY

Bacterial infection by phages was assessed using spot plate assays with phage titers of 10^8 PFU/mL. Briefly, 10 μL of phage lysate was spotted onto an LM17 soft agar overlay containing 10 mM $CaCl_2$ and inoculated with 1×10^8 CFU/mL of the host organism. Spot plates were allowed to dry before incubation aerobically, at 30°C for 24 h.

PHAGE ISOLATION AND PROPAGATION

Raw sewage samples were collected from the sewage treatment facility in Mitchelstown, Co. Cork, Ireland. Phage isolation was conducted as described previously (Alemayehu et al., 2009). Individual plaques isolated following this method underwent 3 successive rounds of plaque purification, to ensure that a pure phage was isolated. Briefly, a single plaque was aseptically removed from an overlay plate using a sterile 1 mL pipette tip and added to 5 mL of mid-log phase host, containing 10 mM $CaCl_2$. Following overnight incubation at 30°C, the mixture was centrifuged at 4,500 rpm for 15 min and filtered through a 0.45 μm pore filter (Sarstedt, Wexford, Ireland). The filtrate was diluted to 10^{-8} and plaqued on the appropriate media for 24 h at 30°C. These steps were repeated twice on the resulting plaques until a pure phage was obtained.

LYTIC SPECTRUM AND ADSORPTION TO LACTOCOCCAL CELLS

The lytic spectrum of ΦL47 was determined, as for dairy lactococcal phages, by spotting 10 μL of phage lysate containing 10^7 PFU/mL onto soft agar seeded with a *L. lactis* strain. Adsorption of phage particles to lactococcal cells was determined as follows: 10 μL of phage lysate ($\sim 10^7$ PFU/mL) was mixed with 2 mL of late exponential cells. Samples were incubated at 30°C

for 10 min with shaking, to allow the phage particles to attach. Each sample was centrifuged at 14,000 rpm for 5 min and filtered through a 0.2 μm filter (Sarstedt, Wexford, Ireland). The number of phages in the supernatant was determined by plaque assay and % adsorption was calculated using the formula [(initial titer—titer in supernatant)/ initial titer × 100%].

ELECTRON MICROSCOPY

High titer phage suspensions for visualization of phage particles were prepared using CsCl gradient purification and ammonium acetate concentration. Pure phage samples were obtained using a CsCl gradient of polyethylene glycol (Sigma-Aldrich, Dublin, Ireland) precipitates as described by Sambrook and Russell (2001). For ammonium acetate precipitation, 1 L of fresh lysate was centrifuged at 10,000 rpm at 4°C for 10 min and filter sterilized using a 0.45 μm pore filter (Sarstedt). Phage particles were subsequently precipitated by centrifugation at 20,000 rpm for 1 h at 4°C. The supernatant was removed and the phage pellet re-suspended in 10 mL of ice cold 0.1 M ammonium acetate (Sigma-Aldrich). After pooling of phage samples, a further 10 mL of ammonium acetate was added and the sample centrifuged at 20,000 rpm for 1 h. The supernatant was again removed and the pellet suspended in a final volume of 1 mL ammonium acetate. Negative staining was performed on both phage samples using 2% uranyl acetate on carbon films. Each grid was examined at an 80 kV acceleration voltage using a Tecnai 10 transmission electron microscope (FEI Company, Eindhoven, The Netherlands). Micrograph images were captured using a MegaView 2 CCD-camera (Olympus SIS, Münster, Germany). Phage structure dimensions were determined based on the average of 10–15 measurements.

STRUCTURAL ANALYSIS OF PHAGE PROTEINS

Analysis of structural proteins was performed as described previously (Kelly et al., 2012) using high titer phage suspensions obtained from ammonium acetate concentration. Samples were mixed with 4X sample loading buffer and heated at 95°C prior to loading in a 12% SDS polyacrylamide gel. Protein bands were stained using Coomassie blue staining and excess dye removed using a de-staining solution (40% ethanol, 53% distilled water and 7% acetic acid). Protein size was estimated using a broad range protein ladder (New England Biolabs, Hertfordshire, UK) as a relative molecular weight marker (MWM).

DNA SEQUENCING, ANNOTATION AND COMPARATIVE ANALYSIS

Bacteriophage DNA sequencing was performed using the Roche GS FLX+ system to >20× coverage (MWG, Ebersberg, Germany). The quality of the raw assembly reads were visualized and verified using the programme Hawkeye (Amos) (Schatz et al., 2013) and Consed (Gordon, 2003). To verify the genome structure, PCR amplicons were generated with the Platinum Hi-fidelity PCR Supermix (Invitrogen, Life Technologies, Dublin, Ireland) for various regions of the genome and at contig ends, followed by direct sequencing. The final phage genome was assembled using the Phred-Phrap-Consed package (Ewing and Green, 1998; Gordon, 2003). ORFs were predicted using the programs prodigal (Hyatt et al., 2010) and Glimmer (Delcher et al., 1999). Annotation was providedby the RAST annotation software (Aziz et al., 2008) and GAMOLA (Altermann and Klaenhammer, 2003). Genome annotation was verified manually using Artemis (http://www.sanger.ac.uk/resources/software/artemis/) (Rutherford et al., 2000) and detected open reading frames (ORFs) were functionally annotated using BLASTp (Altschul et al., 1990). Conserved domains were detected using InterProScan (http://www.ebi.ac.uk/InterProScan/) and DELTA-BLAST (Domain Enhanced Lookup Time Accelerated BLAST) (Marchler-Bauer et al., 2011). Identification of tRNAs was achieved using the software packages tRNA scan-SE (Lowe and Eddy, 1997) and ARAGORN v1.2.36 (Lowe and Eddy, 1997; Laslett and Canback, 2004). Comparative genome analysis of the *Lactococcus* ΦL47 with its most similar relative, *Lactococcus* Φ949, was performed using the Artemis Comparison Tool (ACT) programme (Carver et al., 2005). The genome sequence of ΦL47 is available from GenBank/EMBL under the accession number KF926093.

RESULTS AND DISCUSSION

ISOLATION OF ΦL47 FROM SEWAGE

Previous work in our laboratory led to the isolation of a bank of lactococcal strains from non-dairy origins, including grass, vegetable matter and bovine rumen samples. These strains, which included both *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris*, were evaluated for their flavor-forming ability in a mini-Gouda cheese model and were shown to have potential to diversify flavor in this system (D. Cavanagh, unpublished). Future use of these strains in a commercial setting will depend on a number of technological characteristics inherent in the strains, including their sensitivity to phages. The phage sensitivity of the non-dairy isolate bank was assessed using 8 common dairy lactococcal phages. Phage titers in excess of 10^7 PFU/mL were propagated and tested against the target strain using a spot plate assay performed in triplicate (**Table 1**). ΦPOO8, ΦbIL170, ΦHP, ΦC2 and ΦKSY1 were incapable of infecting all of the non-dairy isolates tested. However, ΦML3 was capable of infecting strains DPC6855 and DPC6856, isolated from grass and bovine rumen, respectively, while ΦebI infected the strains DPC6854 and DPC6855 but not DPC6856 (**Table 1**).

Overall, the non-dairy isolates displayed significant levels of insensitivity to the dairy phages. Given the high evolution rate of phages to overcome bacterial defence systems (Sturino and Klaenhammer, 2006), however, it is highly likely that over time, phages would emerge to threaten these bacterial strains. To understand the interaction of these isolates with the phages that infect them, a screening programme was initiated to identify phages specific for some of the non-dairy lactococci. Following 3 enrichment cycles of raw sewage samples with the grass isolate *L. lactis* DPC6860 as a host, phage ΦL47 was isolated. ΦL47 was found to form clear plaques of ∼0.6 mm diameter when plaqued on *L. lactis* DPC6860. The formation of clear plaques would suggest that ΦL47 is virulent. Phage titers of 10^7–10^8 PFU/mL were recovered after a single propagation.

LIMITED HOST RANGE OF *L. lactis* DPC6860

In order to determine the host range of ΦL47, a range of lactococcal strains were chosen for analysis including industrial dairy

Table 1 | Phage resistance profile of dairy lactococcal phages to non-dairy *Lactococcus* strains.

Strain		Phage							
	Phage group	936				c2			KSY1
	Origin	Φ712	ΦPOO8	ΦblL170	ΦHP	ΦC2	Φebl	ΦML3	ΦKSY1
L. lactis ssp. *lactis* DPC6853	Corn	−	−	−	−	−	−	−	−
L. lactis ssp. *cremoris* DPC6854	Grass	−	−	−	−	−	+	−	−
L. lactis ssp. *cremoris* DPC6855	Grass	−	−	−	−	−	+	+	−
L. lactis ssp. *cremoris* DPC6856	Rumen	+	−	−	−	−	−	+	−
L. lactis ssp. *cremoris* DPC6857	Grass	−	−	−	−	−	−	−	−
L. lactis ssp. *cremoris* DPC6858	Grass	−	−	−	−	−	−	−	−
L. lactis ssp. *cremoris* DPC6859	Grass	−	−	−	−	−	−	−	−
L. lactis ssp. *cremoris* DPC6860	Grass	−	−	−	−	−	−	−	−

starters, strains of non-dairy origins and a strain from raw milk (**Table 2**). It was found that ΦL47 is capable of infecting 3 out of 7 non-dairy *Lactococcus* isolates but only 4 out of the 19 dairy strains, corresponding to hosts of 2 phage groups including the 949 group (**Table 2**). ΦL47 was unable to infect *L. lactis* IL1403, another host strain of Φ949. Contrastingly, although Φ949 was only able to infect 7 of 59 strains of *Lactococcus*, these corresponded to hosts belonging to 5 phage groups (Samson and Moineau, 2010). Levels of adsorption of ΦL47 to *L. lactis* DPC6860 were >85% compared to <40% for other strains tested (**Figure 1**). However, ΦL47 was capable of lysing strain ML8 but not 303 even though a higher level of phage adsorption was observed in the latter. This would suggest that *L. lactis* 303 possesses a form of anti-phage defence which prevents phage DNA entry or cleaves phage DNA.

Table 2 | Host range of *Lactococcus* phage ΦL47.

Species	Origin/Use	Host celllysis
L. lactis ssp. *cremoris* DPC6855	Grass	−
L. lactis ssp. *cremoris* DPC6859	Grass	+
L. lactis ssp. *cremoris* DPC6854	Grass	−
L. lactis ssp. *cremoris* DPC6858	Grass	+
L. lactis ssp. *cremoris* DPC6857	Grass	+
L. lactis ssp. *cremoris* DPC6856	Rumen	−
L. lactis ssp. *lactis* DPC6853	Corn	−
L. lactis ssp. *cremoris* HP	Dairy	+
L. lactis ssp. *cremoris* IE16	Dairy	−
L. lactis ssp. *cremoris* MG1363	Dairy	−
L. lactis ssp. *lactis* IL1403	Dairy	−
L. lactis SMQ562	Dairy	−
L. lactis ssp. *cremoris* UC509	Dairy	−
L. lactis ssp. *lactis* biovar diacetylactis F7/2	Dairy	−
L. lactis ssp. *lactis* ML8	Cheese	+
L. lactis ssp. *cremoris* H88M1	Raw milk	−
L. lactis ssp. *lactis* 303	Cheese	−
L. lactis HT−2	Dairy	−
L. lactis ssp. *cremoris* SK11	Cheese	−
L. lactis U53	Dairy	+
L. lactis ssp. *lactis* biovar diacetylactis DRC3	Dairy	−
L. lactis 83	Dairy	−
L. lactis BA1	Dairy	−
L. lactis ssp. *lactis* biovar *diacetylactis* 938	Dairy	−
L. lactis ssp. *cremoris* 310	Cheese	+
L. lactis ssp. *lactis* 229	Cheese	−
Lactobacillus plantarum	Grass	−
Lactobacillus brevis	Grass	−

EXTENDED TAIL FIBER OF ΦL47

Visualization of phage particles was achieved by transmission electron microscopy performed on samples obtained from both CsCl gradient purification of PEG precipitations and ammonium acetate concentration. Images generated from samples obtained by CsCl gradient showed the majority of phage virions to have disintegrated, with broken tails and empty capsids. Images generated from ammonium acetate preparations displayed intact phage particles which allowed for the estimation of capsid diameter and tail length (**Figure 2**). It was determined that ΦL47 possesses an icosahedral capsid (diameter 75 nm) and a non-contractile tail (length 480 nm; width 15 nm) indicating that this phage is a member of the family *Siphoviridae* of the class *Caudovirales*. Morphologically, ΦL47 is very similar to the other lactococcal phage, Φ949 which possesses a non-contractile tail of 500 nm in length with an icosahedral capsid of 79 nm in diameter (Jarvis, 1984; Deveau et al., 2006; Samson and Moineau, 2010). However, ΦL47 appears to possess a distinctive tail fiber of 280 nm in length and tail fiber width of <10 nm (**Figure 2B**). *Siphoviridae* phages frequently possess a tail fiber involved in phage infection, however, a tail fiber of this length has not previously been reported in lactococcal phages (Vegge et al., 2005; Boulanger et al., 2008; Jakutytė et al., 2012). *Enterococcus faecalis* bacteriophage ΦEF24C-P2 was found to possess a long tail fiber which was associated with higher infectivity against bacterial strains than the un-mutated phage which does not have a tail fiber (Uchiyama et al., 2011). Similar to ΦEF24C-P2, an extended tail fiber was found in *Bacteroides fragilis* phage ATCC 51477-BI, which was isolated from wastewater (Hawkins et al., 2008). No bouquet-like arrangement was identified in ΦL47, as demonstrated by phage Φγ of *Bacillus anthracis* which possessed a 63 nm long tail fiber (Schuch and Fischetti, 2006).

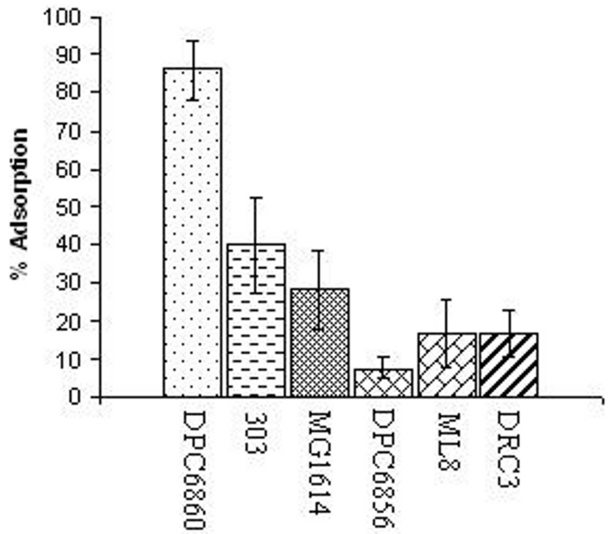


FIGURE 1 | Relative % adsorption of ΦL47 to *Lactococcus* strains of different environmental niches and subspecies designation.

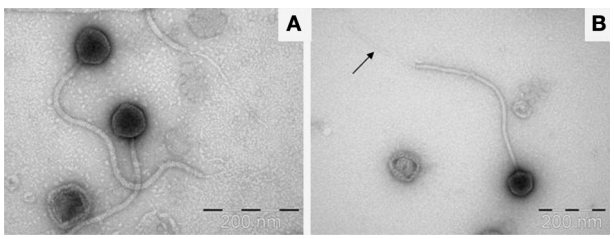


FIGURE 2 | (A) Transmission electron micrograph image of ΦL47 negatively stained using 2% uranyl acetate. **(B)** Single view of ΦL47. Arrow shows the elongated tail fiber.

STRUCTURAL PROTEIN ANALYSIS

SDS-PAGE was employed to isolate the structural proteins of ΦL47 and this led to the identification of 9 individual protein bands (**Figure 3**). Five proteins of low molecular weight (<30 kDa) identified from genome sequence analysis, were not identified on the gel. These proteins may not have been present at a sufficient concentration to allow for visualization. Molecular masses of proteins estimated by SDS-PAGE corresponded to the estimated molecular weight of structural proteins predicted from genome sequencing. Three major structural proteins were identified from SDS-PAGE, with the largest having a molecular weight of 31.4 kDa and may correspond to a base plate protein (ORF 176). One large minor protein of ∼100 kDa was visualized on the gel but did not correspond to any identified ORF. Multiple bands were observed at ∼50 kDa and ∼30 kDa which could not be assigned to identified ORFs. It is possible that these proteins are proteolytic products of some other phage structural proteins, or they are of bacterial origin. Techniques such as N-terminal sequencing of protein bands would allow for a more definite relationship to be established between genome analyses and SDS-PAGE.

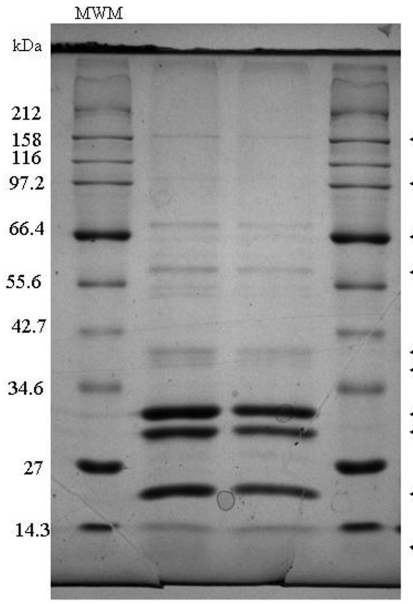
ANALYSIS OF THE L47 GENOME

GENERAL CHARACTERISTICS

To date, all 59 sequenced *L. lactis* phages available in the public domain possess a dsDNA genome and are predominately 15 to 35 kb in size with the exception of the larger genomes of ΦKSY1 (79.2 kb), ΦP087 (60.1 kb), and Φ949 (114.8 kb). Analysis of the ΦL47 genome revealed a dsDNA molecule of 128, 932 bp, making it the largest sequenced lactococcal phage to date. ΦL47 was found to possess a molecular GC content of 32.5% which corresponds to that observed in the similarly sized *Lactococcus* phage, Φ949 (32.7%). The GC content of the host organism, *L. lactis* DPC6860, was calculated as 35.56%. A total of 190 ORFs were identified in the ΦL47 genome, 117 of which shared significant homology with *L. lactis* Φ949 (>50% amino acid identity). The majority of these genes were involved in capsid and tail morphogenesis as well as various hypothetical proteins. Φ949 was isolated from cheese whey in New Zealand over 35 years ago while ΦL47 was isolated from raw sewage indicating how this phage group thrives in their respective environments. Five ORFs were identified that shared no significant identity with proteins in the databases. Similar to the unusual genomic arrangement of Φ949, 4 gene clusters were identified in the ΦL47 genome, with 2 clusters transcribed in the opposite direction. Large inter-genic regions were identified between ORFs 37–38 and ORFs 54–55 which were preceded by a shift in the direction of gene transcription. The 190 ORFs identified in the ΦL47 genome (**Table 3**) account for 88% of the genome which is the same as that identified in Φ949. No putative function could be assigned to 132 ORFs (69.47%). The predominant start codon (89.71%) was ATG with only 8 ORFs starting with an uncommon start codon (TTG, GTG).

TAIL MORPHOLOGY

As stated previously, ΦL47 possessed a long non-contractile tail of 480 nm in addition to a long tail fiber of 280 nm. Both capsid and tail structural genes were encoded from ORFs 157 to 182, and organized in a modular arrangement located downstream from the holin gene (**Figure 4**). The products of 14 of these ORFs encoded putative structural proteins sharing >70% amino acid identity with Φ949 (**Table 3**). This is not unusual as the major structural proteins are generally somewhat conserved in related phages (Suyama and Bork, 2001; Ceyssens et al., 2011). In contrast, proteins involved in adsorption to host cells, such as tail fibers, are expected to differ to a larger degree as they are modified to complement with surface receptors of bacterial hosts (Sandmeier et al., 1992; Hatfull, 2002; Desplats and Krisch, 2003; Silhavy et al., 2010). ORFs 160–163, located within the structural module, encode proteins sharing a high percentage identity (>60%) to proteins of ΦbIL286. ORF 159 encodes a protein with a high percentage identity to a putative tail protein of *Lactococcus* Φ949 but also the putative base plate protein of *Lactococcus* ΦP335, and the phage tail assembly protein of *L. lactis* ssp. *cremoris* A76. A conserved domain search of the putative tail protein of Φ949 reveals the presence of a prophage tail super family cl12123 domain which may possibly act as an endopeptidase.



Putative function	Molecular mass (kDa)	ORF
Tape measure protein	357.8	166
Tail protein	156.2	164
–		
Tail protein	65.9	157
Structural protein	58.1	182
Structural protein	46.9	181
Host specificity	41.8	160
Structural protein	38.3	179
Base plate	31.4	159
Tail component	28.6	165
Structural	28.5	175
Structural	25.1	172
Structural protein	22.9	174
Structural protein	19.3	180
Structural protein	18.7	168
Structural protein	17.5	158
Structural protein	16.9	177

FIGURE 3 | Analysis of the structural proteins of ΦL47 obtained from ammonium acetate precipitations. A broad range ladder (New England Biolabs) was used as a relative molecular weight marker (MWM). Molecular masses of structural proteins was predicted from genome sequence data using the Compute pI/ Mw tool (Gasteiger et al., 2005).

ORF 160 encodes a putative host-specificity protein sharing a 60% identity at the amino acid level with that of the prophage ΦbIL286. This ORF also shares identity to the phage receptor binding protein (35%) and tail host specificity protein (36%) of the plant strain *L. lactis* KF147. ORFs 162 and 163 also indicate homology with ORF 55 of ΦbIL286, both with 89 and 94% identity, respectively. No conserved domains were identified within these two proteins; however, using $BLAST_p$, both proteins were found to possess some identity to a putative tail fiber of *Bacillus* ΦphiNIt1. In order to assign a putative function to these ORFs of ΦL47, ORF 55 of ΦbIL286 was analyzed by InterProScan. The C-terminal region of this gene product was predicted to have an immunoglobulin-like domain IPR007110 as found in the major tail subunit of Enterobacteria ΦHK97 (Juhala et al., 2000) and other phages. Immunoglobulin-like domains participate in various functions, including cell-cell recognition and cell-surface receptors. Therefore, it is likely that these genes also form part of the ΦL47 tail.

HOST CELL LYSIS

In dsDNA phages, the combined action of the holin-lysin genes function in the release of new phage particles from an infected cell (Daniel et al., 2007). ORF 69 was found to encode a putative endolysin sharing 89% identity with that of Φ949, which possesses an amidase_2 domain pfam01510. This ORF also shows a high degree of similarity with gp073, sequence ID YP_001469072.1, of the temperate lactococcal phage ΦKSY1 (83% identity). No holin gene was identified in the same locus as the endolysin, however, a putative holin was located remotely from the endolysin (**Figure 4**). ORF 154 exhibits an 87% identity to the putative phage holin from Φ949, containing a holin LLH superfamily cl09890 domain, a conserved domain of ∼100 amino acids found in prophage and phage of Gram-positive bacteria. The arrangement of the holin and endolysin genes in the ΦL47 genome is not typical of lactococcal phages but a similar organization has been reported in Φ949, ΦP087, and Φ1708 (Garneau et al., 2008; Villion et al., 2009).

DNA REPLICATION AND NUCLEOTIDE BIOSYNTHESIS

The DNA replication module was located downstream from the endolysin gene and upstream from the holin gene. The predicted gene products for ORFs 75 (Helicase), 76 (Primase), 66 (Nucleotidase), 110 and 111 (DNA gyrase) and 114 (Deoxyribosyltransferase) displayed a large degree of homology with Φ949. ORF 89 was identified as a putative replication protein which shares 84% identity with the putative replication initiator protein of *L. lactis* ssp. *lactis* IO-1 (Kato et al., 2012). A DHH domain pfam01368 was identified in ORF 77 which includes the single-stranded DNA exonuclease RecJ. The protein encoded by this ORF exhibits a 95% identity with the single-stranded DNA exonuclease of *Lactococcus* ΦC13, *Lactococcus* ΦCaesusJM1 and *Lactococcus* Φ949. An exonuclease functions to catalyse the cleavage of a single nucleotide from the end of a polynucleotide chain and is involved in DNA repair, recombination and replication (Ceska and Sayers, 1998). No putative endonucleases were identified in the L47 genome, in contrast to two distinct HNH endonucleases in Φ949. Structure specific endonucleases cut at particular DNA structures and in some cases can give a competitive advantage to a given bacteriophage in a mixed infection (Goodrich-Blair and Shub, 1996). It is possible that ΦL47 possesses no HNH endonucleases as it has acquired other characteristics, such as possessing a reduced number of endonuclease

Table 3 | Predicted open reading frames (ORFs) of ΦL47[a,b] and predicted database matches.

ORF	Start	Stop	F/R	MW(kDa)	Best match	% ID	*E*-value	Accession number[c]
1	146	1165	F	39.0	Putative integrase-recombinase [*Lactococcus* Φ949]	324/339 (96)	0.0	YP_004306161.1
2	1174	3495	F	87.6	Putative exodeoxyribonuclease *[Lactococcus* Φ949]	707/772 (92)	0.0	YP_004306162.1
4	4260	4949	F	24.0	Putative transglycosylase [*Lactococcus* Φ949]	195/231 (84)	4e-132	YP_004306164.1
5	4963	5517	F	21.4	Putative uncharacterized protein [Firmicutes bacterium CAG:882]	29/89 (32)	1.8	CDD64083.1
12	7633	8229	F	22.7	Putative thymidine kinase [*Lactococcus* Φ949]	184/196 (94)	3e-134	YP_004306171.1
14	8674	8817	F	5.55	Hypothetical protein, unlikely [*Trypanosoma congolense* IL3000]	16/33 (48)	5.8	CCD16863.1
15	8841	8951	F	5.46	Transglycosylase-like domain protein [*Streptococcus ictaluri*]	18/44 (41)	0.32	WP_008087686.1
23	10569	10796	F	8.43	Phage protein [*L. lactis* ssp. *lactis* KF147]	35/64 (55)	5e-18	YP_003353508.1
24	10814	11191	F	14.0	Hypothetical protein PelgB_21892 [*Paenibacillus elgii* B69]	61/122 (50)	1e-33	ZP_09077133.1
26	11447	11770	F	12.4	Hypothetical protein LACR_2119 [*L. lactis* ssp. *cremoris* SK11]	81/120 (68)	4e-46	YP_811707.1
27	11770	12045	F	10.1	Hypothetical protein ul36_25 [*Lactococcus* Φul36]	50/84 (60)	3e-20	NP_663659.1
28	12038	12367	F	12.5	ORF23 [*Lactococcus* ΦTP901-1]	39/89 (44)	9e-8	NP_112686
29	12364	12609	F	88.9	Hypothetical protein Q33_0026 [*Lactococcus* ΦQ33]	77/81 (95)	3e-50	AFV51055
30	12644	12835	F	7.07	Hypothetical protein [*Lactococcus* Φp2]	38/64 (59)	1e-14	ACI94897.1
31	12912	13415	F	19.8	Hypothetical protein [*Limnohabitans* sp. Rim47]	20/93 (22)	0.32	WP_019430841.1
32	13415	13630	F	8.27	Hypothetical protein [*Methyloversatilis universalis*]	15/48 (31)	2.0	WP_018228448.1
35	14561	14914	F	13.9	Orf28 [*Lactococcus* ΦbIL286]	35/108 (32)	0.37	NP_076662.1
37	15241	15360	F	4.67	Hypothetical protein [*L. lactis* ssp. *lactis* CV56]	24/35 (69)	1e-06	YP_005869010.1
39	17948	17772	R	6.99	Unknown			
40	18472	18083	R	15.0	Hypothetical protein [*Enterococcus* ΦSAP6]	46/118 (39)	6e-11	AEM24750.1
44	19772	19539	R	9.23	Hypothetical protein ACD_5C00356G0007 [uncultured bacterium]	18/61 (30)	1.3	EKE24920.1
45	20124	19852	R	10.9	Hypothetical protein ECED1_3531 [*Escherichia coli* ED1a]	24/69 (35)	0.002	YP_002399391.1
46	20281	20141	R	5.35	Unknown			
47	20856	20716	R	4.52	Unknown			
48	21121	20867	R	9.73	Hypothetical protein EFP_gp119 [*Enterococcus* ΦphiEF24C]	45/79 (57)	1e-19	YP_001504228.1
49	21414	21178	R	9.60	Hypothetical protein [*Enterococcus faecium*]	38/74 (51)	2e-14	WP_002350596.1
52	22318	22007	R	11.7	Hypothetical protein EFP_gp116 [*Enterococcus* ΦphiEF24C]	29/ 72 (40)	4e-12	YP_001504225.1
53	22680	22411	R	10.4	Hypothetical protein LmalK35_10926 [*Lactobacillus mali* KCTC 3596]	25/ 75 (33)	5e-04	ZP_09449152.1
54	23092	22751	R	13.3	LamB/YcsF family protein [*Mesoflavibacter zeaxanthinifaciens* S86]	28/ 122 (23)	1.6	ZP_09499326.1
55	25273	25410	F	5.59	Hypothetical protein ARALYDRAFT_900440 [*Arabidopsis lyrata*]	16/40 (40)	0.63	XP_002886300.1
57	25619	25768	F	5.61	Zinc finger protein 263 [*Monodelphis domestica*]	17/42 (40)	0.34	XP_001370637.2
58	25765	26346	F	22.6	Hypothetical protein [*Streptococcus henryi*]	90/187 (48)	2e-44	WP_018165624.1
60	26593	26979	F	15.8	Hypothetical protein CLJ_0158 [*C. botulinum* Ba4 str. 657]	39/130 (30)	5e-07	YP_002860465.1
61	27057	27260	F	7.92	Hypothetical protein CAEBREN_29888 [*Caenorhabditis brenneri*]	19/53 (36)	0.45	EGT53499.1
62	27257	27691	F	16.8	Hypothetical protein EfaCPT1_gp44 [*Enterococcus* ΦEfaCPT1]	41/139 (29)	6e-04	AFO10841.1

(Continued)

Table 3 | Continued

ORF	Start	Stop	F/R	MW(kDa)	Best match	% ID	*E*-value	Accession number[c]
64	28027	28230	F	7.90	Hypothetical protein LPV712_gp041 [*Lactococcus* Φ712]	34/66 (52)	4e-13	YP_764301.1
66	28820	29419	F	23.2	Putative nucleotidase [*Lactococcus* Φ949]	190/199 (95)	5e-136	YP_004306212.1
69	30662	31708	F	37.8	Putative endolysin [*Lactococcus* Φ949]	311/348 (89)	0.0	YP_001469072.1
72	33459	33629	F	6.35	Hypothetical protein xccb100_2160 [*Xanthomonas campestris* pv. *campestris* str. B100]	15/53 (28)	1.5	YP_001903564.1
75	35411	36955	F	59.2	Putative replicative DNA helicase [*Lactococcus* Φ949]	481/514 (94)	0.0	YP_004306220.1
76	36966	38015	F	40.5	Putative DNA primase [*Lactococcus* Φ949]	302/329 (92)	0.0	YP_004306221.1
77	38022	39806	F	68.0	Putative single-stranded DNA specific exonuclease [*Lactococcus* ΦCB13]	566/593 (95)	0.0	ACU46843.1
80	40336	40815	F	18.2	Membrane protein containing HD superfamily hydrolase domain, YQFF ortholog [*Caloramator australicus* RC3]	27/71 (38)	1.1	CCC58155.1
82	41311	41496	F	7.74	Unknown			
83	41493	41636	F	4.99	Unknown			
84	41639	41854	F	8.84	GJ15471 [*Drosophila virilis*]	20/54 (37)	0.34	XP_002060057.1
85	41848	42081	F	9.16	Conserved hypothetical protein [*Anaerococcus tetradius* ATCC 35098]	16/54 (30)	1.3	ZP_03930743.1
86	42081	42458	F	14.4	GA28089 [*Drosophila pseudoobscura pseudoobscura*]	21/54 (39)	0.44	XP_002133330.1
88	42897	43262	F	14.4	Putative phage protein [*L. lactis* ssp. *lactis* A12]	60/99 (61)	1e-24	CDG04128.1
89	43259	43648	F	14.6	Putative replication initiator protein [*L. lactis* ssp. *Lactis* IO-1]	108/129 (84)	3-75	YP_007508915.1
90	43695	44000	F	12.2	Hypothetical protein [*L. lactis* ssp. *lactis* CV56]	36/105 (34)	6e-07	YP_005868381.1
92	44413	44592	F	7.14	Catechol 1,2-dioxygenase [*Pseudomonas stutzeri* DSM 10701]	23/53 (43)	3.3	AFN77848.1
100	46959	47195	F	8.87	Related to Beta-mannosidase precursor [*Sporisorium reilianum* SRZ2]	15/41 (37)	4.6	CBQ72430.1
105	49552	49986	F	16.8	Hypothetical protein ROI_04910 [*Roseburia intestinalis* M50/1]	21/65 (32)	0.058	CBL07781.1
110	55222	57189	F	73.4	Putative DNA gyrase subunit B-topoisomerase [*Lactococcus* Φ949]	629/651 (97)	0.0	YP_004306254.1
111	57191	59305	F	79.22	Putative DNA gyrase subunit A-topoisomerase [*Lactococcus* Φ949]	675/704 (96)	0.0	YP_004306255.1
114	59918	60436	F	19.0	Putative nucleoside-2-deoxyribosyltransferase [*Lactococcus* Φ949]	166/172 (97)	1e-114	YP_004306259.1
115	60429	61088	F	24.9	Putative nicotinamide mononucleotide transporter [*Lactococcus* Φ949]	205/219 (94)	3e-142	YP_004306260.1
116	61168	63669	F	95.7	Putative anaerobic ribonucleoside-triphosphate reductase [*Clostridium* Φphi8074-B1]	268/791 (34)	2e-134	YP_007237286.1
117	63669	64265	F	22.7	Putative ribonucleoside triphosphate reductase activator small subunit [*Lactococcus* Φ949]	167/198 (84)	2e-126	YP_004306263.1
118	64356	65258	F	35.4	Hypothetical group I intron protein [*Lactococcus* Φ949]	166/238 (70)	8e-111	YP_004306264.1
119	65327	66550	F	45.8	gp109 [*Lactococcus* ΦKSY1]	151/389 (39)	1e-69	YP_001469108.1
122	67083	67322	F	9.16	Putative ribonucleotide diphosphate reductase glutaredoxin subunit[*Lactococcus* Φ949]	71/79 (90)	3e-44	YP_004306267.1
123	67315	67686	F	14.1	Putative ribonucleotide reductase stimulatory protein [*Lactococcus* Φ949]	121/123 (98)	2e-79	YP_004306268.1
124	67700	69889	F	82.8	Ribonucleotide-diphosphate reductase subunit alpha [*Bacillus alcalophilus*]	465/715 (65)	0.0	WP_003323097.1
125	69902	70312	F	16.0	Hypothetical protein TTHERM_00313410 [*Tetrahymena thermophila*]	19/46 (41)	0.48	XP_001033522.1

(Continued)

Table 3 | Continued

ORF	Start	Stop	F/R	MW(kDa)	Best match	% ID	*E*-value	Accession number[c]
126	70395	70991	F	23.0	Hypothetical group I intron protein [*Lactococcus* Φ949]	89/126 (71)	3e-60	YP_004306264.1
127	71101	71751	F	25.7	Hypothetical protein [*L. lactis* ssp. *cremoris* A76]	89/208 (43)	2e-49	YP_005876546.1
128	71773	72513	F	29.3	ATPase [*Solibacillus silvestris* StLB046]	70/229 (31)	8e-18	YP_006461965.1
129	72726	72872	F	5.74	Unnamed protein product [*Tetraodon nigroviridis*]	16/40 (40)	1.2	CAG05661.1
130	72869	73153	F	10.7	M23 peptidase domain-containing protein [*C. perfringens* F262]	29/83 (35)	0.46	EIA16337.1
131	73169	73390	F	8.71	Hypothetical protein LP030nr2_047 [Listeria ΦLP-030-2]	19/37 (51)	3e-04	YP_008126743.1
132	73392	73787	F	15.5	Uncharacterized protein BN765_00487 [*Eubacterium eligens* CAG:72]	27/91(30)	3.1	CDA41513.1
133	73797	74150	F	13.8	Hypothetical protein CRE_21750 [*Caenorhabditis remanei*]	25/79 (32)	1.2	XP_003105387.1
134	74153	74335	F	7.15	Hypothetical protein GY4MC1_0607 [*Geobacillus* sp. Y4.1MC1]	15/48 (31)	1.5	YP_003988046.1
135	74404	75114	F	27.3	Hypothetical protein [*Bacillus amyloliquefaciens* TA208]	85/238 (36)	3e-36	YP_005541036.1
136	75475	76098	F	22.3	Diphosphate reductase [*Streptococcus ovis*]	131/184 (71)	5e-88	WP_018378302.1
137	76177	76746	F	17.0	Hypothetical protein [Lachnospiraceae bacterium A4]	35/142 (25)	0.92	WP_016282214.1
138	76752	77021	F	22.0	Hypothetical protein llmg_2104 [*L. lactis* ssp. *cremoris* MG1363]	27/ 90 (30)	0.008	YP_001033358.1
139	77047	77322	F	10.6	Hypothetical protein [Clostridium sp. 7_2_43FAA]	24/84 (29)	7.2	WP_008676880.1
140	77312	78226	F	10.6	Putative ribonucleotide diphosphate reductase alpha subunit [*Lactococcus* Φ949]]	278/ 298 (93)	0.0	YP_004306269.1
141	78305	78985	F	32.9	Putative Guanylate kinase [*Lactococcus* Φ949]	198/ 226 (88)	6e-144	YP_004306270.1
142	78963	79706	F	27.8	Putative deoxyuridine 5′-triphosphate nucleotidohydrolase [*Lactococcus* Φ949]	239/247 (97)	1e-176	YP_004306271.1
143	79803	80564	F	28.1	Putative protease [*Lactococcus* Φ949]	227/ 253 (90)	1e-167	YP_004306272.1
147	82174	85336	F	122.3	Putative DNA polymerase III alpha subunit [*Lactococcus* Φ949]-truncated	641/ 692 (93)	0.0	YP_004306276.1
148	85420	85596	F	65.1	Putative uncharacterized protein [Odoribacter sp. CAG:788]	14/32 (44)	9.4	WP_021989169.1
153	87639	86959	R	26.1	Putative Holliday junction resolvase [*Lactococcus* Φ949]	208/ 226 (92)	2e-130	YP_004306283.1
154	88100	87693	R	14.7	Putative phage holin [*Lactococcus* Φ949]	118/ 135 (87)	1e-77	YP_004306284.1
157	90418	88586	R	65.9	Putative tail protein [*Lactococcus* Φ949]	468/ 616 (76)	0.0	YP_004306287.1
158	90891	90418	R	17.5	Putative tail protein [*Lactococcus* Φ949]	150/157 (96)	5e-93	YP_004306288.1
159	91768	90932	R	31.4	Putative tail protein [*Lactococcus* Φ949]	134/232 (58)	2e-75	YP_004306288.1
160	92858	91731	R	41.8	Host-specificity [*Lactococcus* ΦbIL286]	136/228 (60)	3e-67	NP_076691.1
161	93009	92836	R	6.4	Orf56 [*Lactococcus* ΦbIL286]	52/57 (91)	1e-29	NP_076690.1
162	93704	93009	R	27.4	Orf55 [*Lactococcus* ΦbIL286]	209/228 (92)	4e-141	NP_076689.1
163	94137	93751	R	13.2	Orf55 [*Lactococcus* ΦbIL286]	120/128 (94)	5e-62	NP_076689.1
164	98313	94153	R	156.2	Putative tail protein [*Lactococcus* Φ949]	1088/ 1309 (83)	0.0	YP_004306288.1
165	99184	98420	R	28.6	Putative phage tail component [*Lactococcus* Φ949]	236/ 254 (93)	3e-169	YP_004306289.1
166	109063	99236	R	357.7	Putative tail tape measure protein [*Lactococcus* Φ949]	2780 /3276 (85)	0.0	YP_004306290.1
167	110073	109081	R	38.4	Putative site specific recombinase [*Lactococcus* Φ949]	326/ 330 (99)	0.0	YP_004306291.1
168	110552	110070	R	18.7	Putative phage structural protein [*Lactococcus* Φ949]	153/ 160 (96)	7e-111	YP_004306292.1
172	112890	112189	R	25.1	Putative phage structural protein [*Lactococcus* Φ949]	197/ 234 (84)	4e-139	YP_004306296.1

(Continued)

Table 3 | Continued

ORF	Start	Stop	F/R	MW(kDa)	Best match	% ID	*E*-value	Accession number[c]
174	114260	113655	R	22.9	Putative phage structural protein [*Lactococcus* Φ949]	186/ 201 (93)	6e-136	YP_004306298.1
175	115012	114260	R	28.5	Putative phage structural protein [*Lactococcus* Φ949]	240/ 250 (96)	2e172	YP_004306299.1
177	115749	115309	R	16.9	Putative phage structural protein [*Lactococcus* Φ949]	143/146 (98)	3e-99	YP_004306301.1
179	117513	116494	R	38.3	Putative phage structural protein [*Lactococcus* Φ949]	318/338 (94)	0.0	YP_004306303.1
180	118088	117552	R	19.3	Putative phage structural protein [*Lactococcus* Φ949]	125/178 (70)	1e-82	YP_004306304.1
181	119358	118117	R	46.9	Putative phage structural protein [*Lactococcus* Φ949]	401/417 (96)	0.0	YP_004306305.1
182	120911	119370	R	58.1	Putative phage structural protein [*Lactococcus* Φ949]	490/513 (96)	0.0	YP_004306306.1
183	122632	120935	R	64.9	Putative terminase ATPase subunit [*Lactococcus* Φ949]	558/565 (99)	0.0	YP_004306307.1
185	125008	123761	R	47.6	Putative DNA polymerase [*Lactococcus* Φ949]	397/415 (96)	0.0	YP_004306309.1
186	126342	125017	R	50.9	Putative phosphate starvation-inducible protein PhoH/ATPase [*Lactococcus* Φ949]	337/441 (76)	0.0	YP_004306310.1

[a] *Hypothetical proteins with >60% identity to Φ949 have been omitted.*

[b] *ORFs shaded gray possess E-values >1.*

[c] *Accession numbers corresponding to the NCBI database.*

Abbreviations: F/ R, Forward/ Reverse; MW, Molecular weight; %ID, % Identity; E-value, Expect-value.

recognition sites, that offer an advantage over other bacteriophages. Group I introns are frequently associated with endonucleases (Chevalier and Stoddard, 2001), however, ORFs 118 and 126 were both found to encode this type of intervening sequence (IVS) between ribonucleotide reductase proteins. Previously, two introns were found within the ribonucleotide reductase large subunit of *Staphylococcus aureus* ΦTwort (Landthaler et al., 2002). In *Escherichia coli* ΦT4, the aerobic Ribonucleotide reductase small subunit and the anaerobic ribonucleotide reductase were both found to possess group I introns (Sjöberg et al., 1986; Young et al., 1994). One explanation offered for the retention of introns in genes, such as those involved in ribonucleotide reduction, is that they encode functions replicated in the host organism that can be utilized for phage survival until such time as the insertion element and host environment adapt to one another (Derbyshire and Belfort, 1998).

The acquisition of ribonucleotide reductases (RNRs) is thought to arise from the host genome, to enable the adaptation to certain environmental conditions (Dwivedi et al., 2013). These enzymes function in DNA replication and repair via the conversion of ribonucleotides to deoxyribonucleotides (Dwivedi et al., 2013). The gene products of 7 ORFs were found to encode ribonucleotide di- and tri-phosphate reductases. ORF 116 encodes a putative anaerobic ribonucleoside-triphosphate reductase with a 25% identity to a putative anaerobic ribonucleoside-triphosphate reductase of *Clostridium* Φphi8074-B1. A class III ribonucleotide reductase domain cd01675 was identified within this gene product which uses a FeS cluster and S-adenosylmethionine to generate a glycyl radical. ORF 124 shares a 65% identity with the ribonucleotide-diphosphate reductase subunit alpha of *Bacillus alcalophilus* and contains a class I RNR domain cd01679. In contrast to class III, a class I RNR domain uses a di-iron-tyrosyl radical. The presence of class I and class III RNR genes in ΦL47 conforms with the RNR complement generally found in phages isolated from sewage (Dwivedi et al., 2013). Other ribonucleotide reductases shared a large degree of homology with Φ949 as well as other genes involved in nucleotide transport, modification and degradation.

tRNA ENCODING GENES IN L47 GENOME

The role of tRNAs in phage genomes is thought to be the encoding of codons that are less frequent in the host genome, thus allowing for the increased expression of phage proteins (Bailly-Bechet et al., 2007). In all, 8 tRNA genes (**Table 4**) were identified over a small region of the genome (48,524–53,674 bp) in ΦL47, using the tRNA-scan SE and Aragorn software (Lowe and Eddy, 1997; Laslett and Canback, 2004). tRNAs had a %GC content ranging from 39.4 to 50.7. Between ORFs 106 and 107, were positioned $tRNA^{Arg}$, $tRNA^{Asp}$ and $tRNA^{Ala}$ while between ORFs 107 and 108 were $tRNA^{Asn}$, $tRNA^{Ser}$ and $tRNA^{Trp}$. A 58 bp intron, from position 34 to 35, was identified in $tRNA^{Ser}$. Two $tRNA^{Met}$ genes were identified, the first situated between ORFs 101 and 102, and the second between ORFs 107 and 108. The possession of more than one tRNA for a specific amino acid is a phenomenon that has been observed in *Lactobacillus plantarum* bacteriophage ΦLP65 (Chibani-Chennoufi et al., 2004b). The acquisition of tRNA genes from the host is proposed as being a random event and the genes are either retained, via a set of selection mechanisms, or they are lost (Bailly-Bechet et al., 2007). It is conceivable that ΦL47 has acquired two $tRNA^{Met}$ in order to gain a fitness advantage,

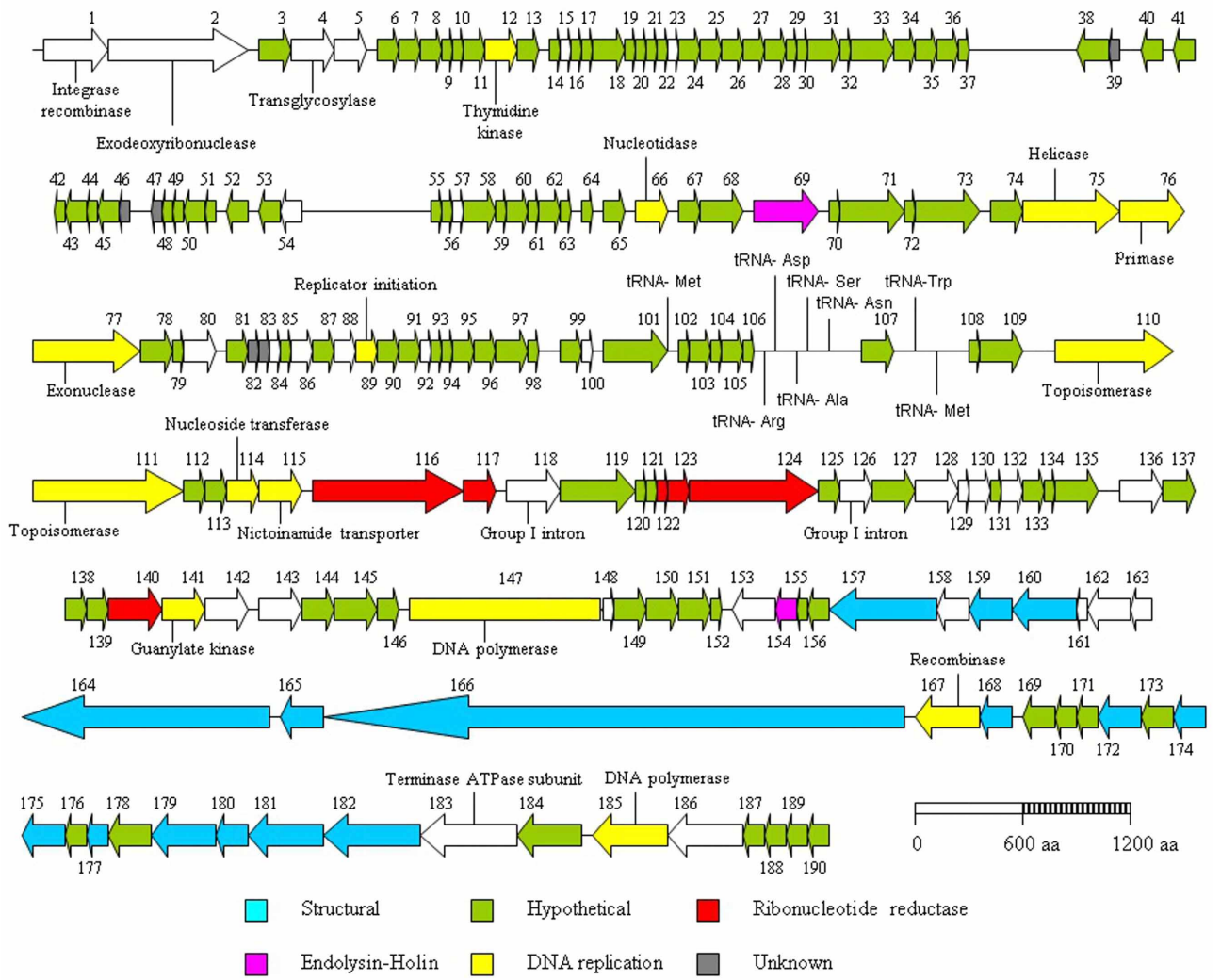


FIGURE 4 | Genome organization of the lactococcal phage ΦL47. Each arrow represents an open reading frame (ORF), the orientation of which shows the direction of transcription. The predicted function of each ORF was determined by bioinformatic analyses (Carver et al., 2005).

due to the relatively harsh environment that it inhabits. *In-situ* burst sizes for organisms in a nutrient-poor environment, such as that from which the host was isolated, are generally smaller than when the host is infected in a nutrient rich, chemically defined media (Chibani-Chennoufi et al., 2004a). The retention of particular tRNA genes in phage genomes are thought to correspond to codons that are less abundant in the host genome (Bailly-Bechet et al., 2007) although this was not observed for Φ949 and its host *L. lactis* IL1403 (Samson and Moineau, 2010). Therefore, we may only hypothesize that these tRNA genes are associated with controlling phage protein production and are possibly involved in increasing reproduction rate and reducing latency time (Bailly-Bechet et al., 2007).

The arrangement of the tRNAs into distinct blocks suggests that they were obtained through separate recombination events with either host DNA, other phages or a combination of both (Weinbauer, 2004). The presence of tRNAs has been found to be particularly common in phages with a larger genome size (Bailly-Bechet et al., 2007). Six tRNA genes were identified in Φ949, the most identified in a lactococcal bacteriophage (Samson and Moineau, 2010). Similarly, in ΦLb338-1 and ΦK, both

Table 4 | tRNA arrangement in ΦL47.

tRNA	Amino acid	Anti-codon	Size (bp)	% GC	Start	End
1	Met	CAT	73	47.9	48524	48596
2	Arg	TCT	73	39.7	50343	50415
3	Asp	GTC	75	50.7	51213	51287
4	Ala	CGC	71	50.7	51639	51709
5	Ser	ACT	92	43.5	51967	52116
6	Asn	ATT	76	39.5	52830	52905
7	Trp	CCA	71	39.4	53114	53184
8	Met	CAT	74	55.4	53400	53473

with a genome larger than 100 kb, 2 and 4 tRNAs were identified, respectively (O'Flaherty et al., 2004; Alemayehu et al., 2009). No tRNA encoding regions were found in 2 recently elucidated lactococcal phage genomes, belonging to a new P335 group, with sizes of ∼31 kb (Mahony et al., 2013). However, 5 tRNA genes have been identified in ΦP087, a P087 species, of a smaller genome size (60,074 bp) than Φ949 and ΦL47 (Villion et al., 2009).

COMPARATIVE GENOME ANALYSIS

Numerous ORFs in the L47 genome were found to show identity to putative proteins of other lactococcal phages. ORFs 27–30 encode hypothetical proteins belonging to different lactococcal phages (**Table 3**). No conserved domains were detected within these ORFs. ORF 119, showing 39% identity with *Lactococcus* ΦKSY1, was found to encode a pfam01139 domain, a conserved domain of the uncharacterized protein family UPF0027. Putative proteins expressed by *Enterococcus* ΦphiEF24C and *Enterococcus faecium* are also found to be similar to those identified in the ΦL47 genome (3 ORFs; >40% identity). Previously, lactococcal ΦP087 was shown to possess genes with a high degree of similarity to structural genes of a prophage of *Enterococcus faecalis* V538 (Villion et al., 2009). Of note, ORF 23 was found to be highly similar to a phage protein of the plant *Lactococcus* strain, KF147. Upstream from the structural genes, ORF 130 encodes a M23 peptidase domain containing protein similar to *Clostridium perfringens* F262 (Nowell et al., 2012). The position of this gene with genes involved in DNA replication, suggests that it is not tail associated as in *Lactococcus* phages Tuc2009 and TP901-1 (Seegers et al., 2004; Stockdale et al., 2013). This peptidase would possibly hydrolyse peptidoglycan via D-Ala–D-Asp endopeptidase activity enabling the penetration of stationary phase lactococcal cells (Samson et al., 2013).

Comparative genomic analysis shows the high similarity at a nucleotide level between the ΦL47 and Φ949 phages (**Figure 5**). In addition, given the close amino acid identity at protein level across the genome (**Table 3**), suggesting a number of shared functions, it is likely that these phages have shared a common ancestor at some point. Studies of the more common lactococcal phage groups have indicated a large degree of homology between members of the c2 and 936 species while the P335 species displays a polythetic species theory (Deveau et al., 2006). Although much discussion surrounds phage evolution, in relation to virulent phages vertical lines of evolution are believed to be crucial in the development of certain phage families (Brussow and Kutter, 2005) and are particularly evident in dairy phages (Brüssow and Desiere, 2001). However, horizontal genetic exchange also plays a key role in the evolution of phages as exemplified by genetic mosaicism formed from a high occurrence of transfer events (Canchaya et al., 2003). Genetic exchange between bacteria and other phages from distinct niches is hypothesized to be pivotal in the evolution of rare lactococcal phages (Fortier et al., 2006; Chopin et al., 2007; Garneau et al., 2008; Villion et al., 2009). Due to the high degree of sequence homology between these two

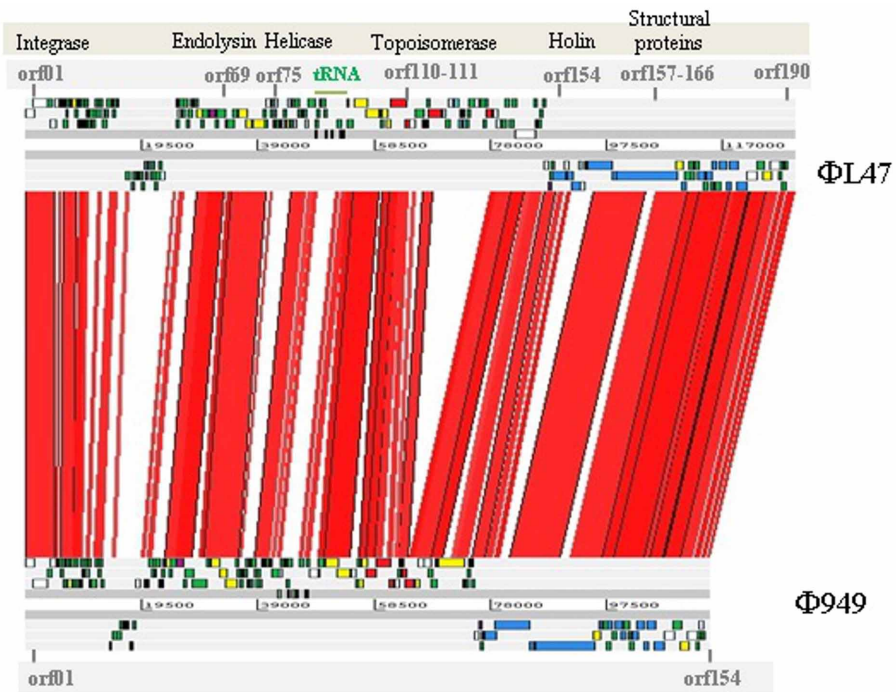


FIGURE 5 | BLASTn-based alignment (nucleotide identity >90% shown) of the genome sequences of *L. lactis* phage ΦL47 and Φ949 as displayed by the Artemis comparison tool (ACT). Red lines between genomes indicate orthologs in the same orientation. Coding sequences are color coded as described in **Figure 4**; structural (blue), hypothetical (green), DNA replication (yellow), ribonucleotide reductase (red), endolysin-holin (mauve), unknown (gray).

phages, it is expected that ΦL47 is another member of the 949 group. Analysis of Φ111 revealed this lactococcal phage to possess a genome size of ∼134 kb and a long tail of 470 nm (Prevots et al., 1990). However, without comparative genome analysis it can only be speculated that Φ111 is a representative of the 949 group.

CONCLUDING REMARKS

To date, numerous studies have investigated dairy lactococcal phages and how they interact with their respective hosts. Industrial *Lactococcus* strains, used in modern cheese production, are thought to have evolved from plant strains (Kelly et al., 2010). ΦL47, isolated from a non-dairy environment, possessed significant similarity to the rare, dairy phage Φ949, with both phages isolated from different environments almost 40 years apart. ΦL47 was found to possess a number of features which differentiate it from Φ949, most notably a long tail fiber, not previously reported in phages of *Lactococcus*. This tail fiber may play an important role in enabling ΦL47 to infect *L. lactis* DPC6860, which was largely resistant to dairy phages, and may account for the persistence of successful virulent phages in the wider environment as observed for ΦQ33 and ΦBM13 (Mahony et al., 2013). Further studies are required to establish the diversity of lactococcal bacteriophages from non-dairy origins and the similarity they may possess with dairy phages of other groups. This information could shed further light on the mechanisms they possess that allow them to thrive in harsher environments and may advance our understanding of host recognition and infection by lactococcal phages.

AUTHOR CONTRIBUTIONS

Daniel Cavanagh isolated phage, performed biological assays, genome analysis, data analysis and drafted the manuscript. Caitriona M. Guinane performed sequence assembly, analysis and assisted in manuscript drafting. Horst Neve performed transmission electron microscopy of phage particles. R. Paul Ross and Aidan Coffey advised on experimental design. Gerald F. Fitzgerald is a supervisor of this project. Olivia McAuliffe. is a supervisor of this project and assisted in the design of experiments, data analysis and manuscript drafting.

ACKNOWLEDGMENTS

This work was supported by the Irish Dairy Levy Research Trust. Daniel Cavanagh was supported by the Teagasc Walsh Fellowship Programme.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 October 2013; accepted: 18 December 2013; published online: 13 January 2014.

Citation: Cavanagh D, Guinane CM, Neve H, Coffey A, Ross RP, Fitzgerald GF and McAuliffe O (2014) Phages of non-dairy lactococci: isolation and characterization of ΦL47, a phage infecting the grass isolate Lactococcus lactis ssp. cremoris DPC6860. Front. Microbiol. ***4****:417. doi: 10.3389/fmicb.2013.00417*

This article was submitted to Virology, a section of the journal Frontiers in Microbiology.

frontiers in MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 13 March 2014
doi: 10.3389/fmicb.2014.00098

Temperate *Streptococcus thermophilus* phages expressing superinfection exclusion proteins of the Ltp type

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Lipoprotein Ltp encoded by temperate *Streptococcus thermophilus* phage TP-J34 is the prototype of the wide-spread family of host cell surface-exposed lipoproteins involved in superinfection exclusion (sie). When screening for other *S. thermophilus* phages expressing this type of lipoprotein, three temperate phages—TP-EW, TP-DSM20617, and TP-778—were isolated. In this communication we present the total nucleotide sequences of TP-J34 and TP-778L. For TP-EW, a phage almost identical to TP-J34, besides the *ltp* gene only the two regions of deviation from TP-J34 DNA were analyzed: the gene encoding the tail protein causing an assembly defect in TP-J34 and the gene encoding the lysin, which in TP-EW contains an intron. For TP-DSM20617 only the sequence of the lysogeny module containing the *ltp* gene was determined. The region showed high homology to the same region of TP-778. For TP-778 we could show that absence of the *attR* region resulted in aberrant excision of phage DNA. The amino acid sequence of mature $Ltp_{TP\text{-}EW}$ was shown to be identical to that of mature $Ltp_{TP\text{-}J34}$, whereas the amino acid sequence of mature $Ltp_{TP\text{-}778}$ was shown to differ from mature $Ltp_{TP\text{-}J34}$ in eight amino acid positions. $Ltp_{TP\text{-}DSM20617}$ was shown to differ from $Ltp_{TP\text{-}778}$ in just one amino acid position. In contrast to $Ltp_{TP\text{-}J34}$, $Ltp_{TP\text{-}778}$ did not affect infection of lactococcal phage P008 instead increased activity against phage P001 was noticed.

Keywords: *Streptococcus thermophilus*, prophage, superinfection exclusion, TP-J34, TP-778L, TP-EW, TP-DSM20617

INTRODUCTION

Superinfection exclusion (sie) is generally known as a mechanism by which a prophage residing in a host cell prevents infection of the lysogenic host cell by other phage through blocking DNA injection (Donnelly-Wu et al., 1993). This protects the host from being lysed by the infecting and multiplying incoming phage, and hence the prophage will not be destroyed in the process of phage multiplication (McGrath et al., 2002; Mahony et al., 2008).

Sie has been mostly described for prophages of Gram-negative bacteria: P22 residing in *Salmonella typhimurium* (Hofer et al., 1995), Lambda-like phages in *Escherichia coli* (Cumby et al., 2012), and kappa-phage K139 in *Vibrio cholerae* (Nesper et al., 1999). Interestingly, sie has also been described for lytic T-even phages of *E. coli* (Lu and Henning, 1994). In Gram-positive bacteria, sie has been identified in prophages of corynebacteria (Groman and Rabin, 1982), *Lactococcus lactis* (McGrath et al., 2002), and *Streptococcus thermophilus* (Sun et al., 2006). One common feature of many of these proteins appears to be their targeting to the external side of the cytoplasmic membrane by either an N-terminal membrane-spanning helix (Mahony et al., 2008; Cumby et al., 2012) or a lipid-anchor (Sun et al., 2006). One exception appears to be the Glo protein of *Vibrio cholerae*, which has been described to a be soluble periplasmic protein (Nesper et al., 1999).

In temperate *S. thermophilus* phage TP-J34, a sie system is encoded by the *ltp* gene, residing within the lysogeny module. *ltp* is transcribed in the prophage state and encodes a lipoprotein, which is tethered to the outside of the cytoplasmic membrane, where it prevents injection of the DNA of the infecting phage into the cytoplasm of the host cell (Sun et al., 2006). Besides its rather weak activity against *S. thermophilus* phages, Ltp shows high activity against lactococcal phage P008 (Sun et al., 2006).

Ltp has been shown to consist of three different functional units: a lipid moiety for membrane anchoring, a serine-rich spacer region, and a repeat domain responsible for sie (Sun et al., 2006; Bebeacua et al., 2013). When expressed without its lipid-anchor, its host-range is extended to phages P335 and P001 belonging to different lactococcal phage species (Bebeacua et al., 2013). Thus, the active domain of Ltp may represent a broad-spectrum phage-resistance protein.

Genes encoding proteins with amino acid sequence similar to Ltp have been found to be scattered among Gram-positive

bacteria and phages. No such gene has been described for *L. lactis* strains and phages, respectively (Sun et al., 2006), although lactococci and streptococci and their phages are very closely related (Proux et al., 2002). Within the 11 publicly available sequenced genomes of *S. thermophilus* phages 2972, 5093, 7201, 858, ALQ13.2, Abc2, DT1, O1205, Sfi11, Sfi19, Sfi21 <http://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?opt=phage&taxid=10239&host=bacteria>, *ltp* determinants have not been identified. Phages O1205 (Stanley et al., 1997) and Sfi21 (Brüssow and Bruttin, 1995) are the only temperate among the 11 phages. However, they are closely related to the virulent *S. thermophilus* phages (Brüssow and Bruttin, 1995; Lucchini et al., 1999; Desiere et al., 2002). They all together may form just one species (Quiberoni et al., 2010). A differentiation of the 11 phages according to their DNA-packaging mechanism resulted in two sub-species (Quiberoni et al., 2010), represented by Sfi21 (*cos*-type) and Sfi11 (*pac*-type) (Proux et al., 2002). O1205 belongs to the *pac*-type (Stanley et al., 1997), indicating that the type of infection is of minor importance for the relatedness of phages.

To investigate the distribution and diversity of members of the Ltp protein family among strains of *S. thermophilus* and to analyze the relatedness of phages carrying an *ltp* gene, we screened among *S. thermophilus* strains for prophages carrying genes similar to *ltp*. For two temperate phages - TP-J34L and TP-778L, we analyzed the whole genome sequences. Of the two other phages, TP-EW and TP-DSM20617, we determined the sequences of some selected DNA regions: *ltp* gene for both phages, lysogeny module for TP-DSM20617, and putative host specificity gene and lysin gene for TP-EW. The two Ltp proteins of phages TP-J34 and TP778 were functionally compared and found to differentially inhibit lactococcal phages. The differences in inhibition are discussed with respect to the differences found in the amino acid sequences of the two Ltp proteins.

MATERIALS AND METHODS

BACTERIA AND PHAGES

S. thermophilus strains used in this study were: J34 (lysogenic wild type), J34-6 (prophage-cured J34), SK778 (lysogenic wild type), DSM20617 (lysogenic wild type, German Collection of Microorganisms and Cell Cultures - DSMZ), and EW (lysogenic wild type).

The following phages were used: TP-J34 (wild type lysate, obtained by induction of the prophage) (Neve et al., 2003), TP-J34L (deletion derivative of TP-J34) (Neve et al., 2003), TP-778 (wild type lysate, obtained by induction of the prophage; this study), TP-778L (single plaque isolate from wild type lysate, this study), TP-DSM20617 (wild type lysate, obtained by induction of the prophage; this study), TP-EW (wild type lysate, obtained by induction of the prophage; this study).

The following lactococcal phages from our collection were used to test for infection-blocking activities of Ltp-derivatives: P197, P220, P624, P653, P684 (c2-species); P955, P957, P983, P993, P996 (936-species); P615 (P335-species). They had been assigned to species by electron microscopic inspection of their morphologies.

GROWTH MEDIA, GROWTH CONDITIONS, PHAGE PROPAGATION, PROPHAGE INDUCTION, PHAGE-CURING, AND RELYSOGENIZATION

S. thermophilus strains were routinely grown at 40°C in modified M17 medium containing lactose (th-LM17) (Krusch et al., 1987). For phage propagation, glycine-lysis medium was used: thM17 supplemented with 8 mM $CaCl_2$ and 1% glycine (Sun et al., 2006). Prophage induction was carried out with UV-light or mitomycin C. For UV-light induction, cells from a growing culture in log-phase were harvested by centrifugation, re-suspended in ½ volume of 0.1 M MgSO4 and pumped through a quartz tube (internal diameter, 1.3 mm; length, 75 cm) placed under a laboratory 254 nm UV lamp (Schütt, Göttingen, Germany) at short distances (maximum 5 cm). Thereafter, the cell suspensions were mixed with another ½ volume of double-concentrated th-LM17 medium and incubated in the dark at 40°C. Induction was considered successful, when complete lysis was seen after ca. 3–4 h. For mitomycin C induction, different concentrations of mitomycin C (between 0.1 and 1 μg/ml) were added to growing cultures at early log-phase. Induction was considered successful, when turbidity increased for ca. 90 min after mitomycin C addition and then dropped to low turbidity levels.

Phage lysates were routinely centrifuged (Beckmann J2-21 centrifuge, 6000 rpm, 20 min, 4°C) and subsequently sterile filtered (nitrocellulose filters, pore size 0.45 μm).

Efficiency of plating was determined as described by Sun et al. (2006). Spot assays for determining the effects of Ltp-derivatives on phage infection were carried out by spotting 10 μl each of serial dilutions of phage lysates on agar plates overlaid with 0.75% top agar seeded with appropriate host bacteria.

All other relevant and specific information can be found in Neve et al. (2003).

DNA TECHNIQUES

Isolation of chromosomal DNA followed the method of Leenhouts et al. (1990) with some modifications. Ten ml th-LM17 medium (supplemented with 40 mM DL-threonine) was inoculated with *S. thermophilus*. Incubation proceeded at 40°C until an optical density at 620 nm (OD_{620}) of ca. 0.8 was reached. From 2 ml of the culture, cells were sedimented by centrifugation (Eppendorf microcentrifuge) and washed once with 2 ml of bi-distilled water. The cells were resuspended in 0.5 ml buffer pH 8.0, containing 20% sucrose, 10 mM Tris-HCl, 10 mM EDTA, 50 mM NaCl, 2.5 mg lysozyme and 30 units mutanolysin. After incubation at 55°C for 10 min, 25 μl of 10% SDS and 60 μl of proteinase K were added. After mixing by inversion, incubation proceeded for 1 h at 60°C. Finally, DNA was taken up in 200 μl Tris-EDTA buffer of pH 8.0.

Phage DNA was isolated from CsCl-purified phage with subsequent phenol extraction following the procedure described by Sambrook and Russel (2000).

Restriction analyses were done according to Sambrook and Russel (2000). Enzymes and recommended buffers were purchased from New England Biolabs (Frankfurt, Germany).

Agarose gel electrophoresis and Southern blot analysis were carried out as described by Sambrook and Russel (2000).

For digoxigenin-labeling of DNA, the "DIG DNA Labeling Kit" of Roche Diagnostics (Mannheim, Germany) was applied, following the manual of the supplier.

PCR was carried out on an Eppendorf Mastercycler 5333 or on a Perkin Elmer GeneAmp PCR System 9600. Primers (**Table 1**) were purchased from MWG Biotech (Ebersberg, Germany). The following pipetting scheme was used: 5 µl 10 × $(NH_4)_2SO_4$ buffer, 5 µl dNTPs (2 mM), 2 µl Tween 20 (2.5%), 1 µl of each of both primers (100 µM), DNA polymerase [10 parts Taq-polymerase (Quiagen, Hilden, Germany) plus 1 part Pfu-polymerase (Stratagene, Amsterdam, The Netherlands), diluted 1:5 with distilled water], 1 µl template-DNA, bi-distilled water 34 µl. PCR was carried out as "hot start" PCR (D'Aquila et al., 1991), starting with 5 min at 95°C for denaturation, holding at 80°C for addition of polymerase, followed by 30 cycles involving denaturation (95°C for 1 min), annealing (at mean Tm of primer pair for 1 min) and elongation (72°C for variable duration: ca. 1 min for 1 kb expected length). Finally, PCR concluded with an elongation at 72°C for 5 min.

An internal 384 bp fragment of *ltp* was amplified by PCR as follows. The reaction solution in the thermal cycler contained 10 µl of 10× PCR kit buffer (Appligene Oncor, USA), 10 µl of dNTP-mix (Appligene Oncor, USA), 4 µl of Tween-20, 1 µl of both primers B and D (100 pmol/ml), 5 µl (0.1 µg) of DNA, 66.5 µl of H_2O and 2.5 µl of Taq DNA polymerase (1 unit/µl, Roche). Negative controls were set up similarly except that template DNA was omitted. Prior to cycling, the reaction mixture was heated to 95°C for 5 min, followed by 35 cycles of 30 s at 95°C, 30 s at 50°C, 30 s at 72°C and a final extension at 72°C for 7 min.

For "long-range" PCR (expected PCR products of up to ca. 4 kb), amplification was done following the "touchdown" protocol of Don et al. (1991). Primer pair D8+ and D12+ was applied. Annealing temperature in the first cycle was 10°C higher than the mean Tm of the primer pair. In the following 29 cycles, annealing temperature was reduced by 0.5°C per cycle. Finally, 10 cycles were added with an annealing temperature °C lower than the mean Tm of the primer pair. Elongation in that case was always 4 min.

Sequencing of the TP-J34 genome was done on a LI-COR 4200 system (MWG Biotech) according to the instructions of the supplier. Sequencing-PCR was done using the "Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (RPN 2438)" (Amersham Pharmacia Biotech, Freiburg, Germany), following the instructions of the supplier. Sequencing primers were labeled with fluorescence dye IRD800 (MWG Biotech). The sequence was completely determined for both DNA strands. It is available under EMBL accession number HE861935.1.

Sequencing of genomic DNA of TP-778L was done by AGOWA (Berlin, Germany) using 454 sequencing with an average coverage of approximately 20 fold. The sequence is available under EMBL accession number HG380752.1

For sequencing of terminal ends of the integrated prophage and host DNA regions flanking the insertion sites, the following primers were applied: primer pair primer4 (targeting the gene encoding 50S ribosomal protein L19) (Bruttin et al., 1997) and int.cro.2 (targeting the *cro* gene of temperate *Streptococcus* phages) for amplification of the left and primer pair lys.1 (targeting the lysin gene of temperate *Streptococcus* phages) and primer 3 (targeting an untranslated DNA region) (Bruttin et al., 1997) for amplification of the right flanking region. Both sequences are available under EMBL accession numbers HG917969 (left) and HG917970 (right).

The sequence of the DSM20617 prophage lysogeny module defined by primers 4 and Mz12.R binding sites was completely determined on both strands by primer walking. The sequence is available under EMBL accession number HG917971.

Table 1 | PCR-primers used for amplification of genomic DNA.

Primer	Sequence [5′→3′]	References
D8	GGGTTGGAGCATTAGAAG	This study
D12	ACCAACTGAAATGCTACC	This study
D8+	GGGTTGGAGCATTAGAAGGTGGATC	This study
D12+	TCCTACCACCAACTGAAATGCTACC	This study
LYSup	GAACGAGCATTGAACTAC	This study
LYSdown	CAGTTCACGATACAGGTC	This study
terS-F	GCTCATTTGTGGGCTGTC	This study
terS-R	CAACGGTCTTACCTGCTC	This study
ltp-F	TAGCAACAGCGTAGTCAGC	This study
pri.C1-R	AAGCAAAGAGGTAGCAGAATC	This study
lys1	CACAAGCCTTAAAAGAGGCA	This study
3	CACAATCCTTCATCAAGC	Bruttin et al., 1997
4	GCAAGGTAAAGCTGCAC	Bruttin et al., 1997
Int.cro.2	TTTTTCTCCCATGCACTAACC	This study
MZ12.R	ATAGCAGATTATCGAATCGGTCAG	This study
8F	AGAGTTTGATCCTGGCTCAG	Beumer and Robinson, 2005
1525R	AAGGAGGTGATCCAGCC	Beumer and Robinson, 2005
B	GGCAAGCTTCGCTCTTGCTTGTTCTC	This study
D	GGCGAATTCTAGCAACAGCGTAGTCAGC	This study

CLONING OF *ltp*$_{TP-778}$

Using primers *ltp*-XbaI and *ltp*-HindIII binding upstream and downstream, respectively, the *ltp*$_{TP-778}$ open reading frame was amplified by PCR. After restriction with the corresponding restriction enzymes the *ltp* orf was ligateded into XbaI/HindIII-cleaved pMG36e. After transformation into *L. lactis* Bu2-60, transformed cells were selected and plasmids extracted. By DNA sequencing plasmid pYAL1-3 was confirmed to be the correct construct.

SEQUENCE ANALYSIS

For identification of open reading frames "*orf* finder" <http://www.ncbi.nlm.nih.gov/gorf/gorf.html> and "Artemis" (Rutherford et al., 2000) were applied. To obtain an overview over the major directions of transcription, only *orfs* with coding capacities larger than 100 amino acids were considered in a first draft. Gaps between *orfs* were inspected for potential *orfs* as small as ca. 50 amino acids by searching for appropriate start codons in connection with potential ribosome binding sites. For annotation

"blast" analyses were performed directly on the genes predicted by "orf finder" or "Artemis."

tRNA genes were searched for by applying the "tRNAscan-SE" program of Lowe and Eddy (1997), and the "Tandem Repeat Finder" (Benson, 1999) was applied for searching for tandem repeats.

Functional assignment of gene products to protein families and identification of motifs of functional significance was done online <http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1> using SMART (Simple Modular Architecture research Tool) (Schultz et al., 1998; Letunic et al., 2009).

Dot plots were performed online <http://www.vivo.colostate.edu/molkit/dnadot/index.html>, (Maizel and Lenk, 1981) with the window size set to 13 and the mismatch limit set to 0.

For multiple sequence alignment, ClustalW at the EMBL-EBI website <http://www.ebi.ac.uk/Tools/msa/clustalw2/> (Larkin et al., 2007) or BLAST <http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq&LINK_LOC=align2seq> (Altschul et al., 1990) was applied.

CRISPR spacer sequences were searched for at the "CRISPRs web server" by blasting phage genomic DNA sequences against the CRISPR database <http://crispr.u-psud.fr/crispr/BLAST/CRISPRsBlast.php> (Grissa et al., 2007).

RESULTS

S. thermophilus temperate phage TP-J34 carrying an *ltp* gene has been described in some detail (Neve et al., 1998, 2003; Sun et al., 2006). Isolation of TP-778 has also been described (Neve et al., 2004). It has been identified as related to but considerably different from TP-J34 by subjecting DNAs extracted from 142 *S. thermophilus* strains and digested by HindIII to Southern blots using digoxigenin-labeled TP-J34 DNA as probe. In a further screening, more than 100 strains were tested by Southern hybridization with a probe generated from the $ltp_{TP\text{-}J34}$ gene using primers B and D. Positive signals were obtained from three strains. Upon induction with mitomycin C two strains gave rise to phages with DNA restriction patterns identical to TP-J34 (data not shown). The third strain, *S. thermophilus* DSM20617, a strain from DSMZ collection which had been included in the screening, had originally been considered non-inducible (Sun, 2002). Only very recently it was shown to harbor an inducible prophage, named TP-DSM20617. TP-EW was identified as an inducible prophage in an *S. thermophilus* strain isolated from German yoghurt. Its DNA was found to give rise to restriction patterns highly similar to those of TP-J34, however, two restriction fragments in the HindIII restriction pattern differed from the TP-J34 pattern (see **Figures 1A,B**).

The morphologies of the three phages, TP-EW, TP-DSM20617, and TP-778L were almost identical to TP-J34 (**Figure 2**), the morphology of which—isometric head and long flexible tail of ca. 250 nm length—has been described already (Neve et al., 2003).

NUCLEOTIDE SEQUENCES

We determined whole genome sequences for TP-J34 and TP-778L. In addition, left and right genome regions flanking prophage TP-778 were sequenced. For TP-EW, the two genome regions differing from those of TP-J34 (*orf48* and the lysin gene) were sequenced in addition to the *ltp* gene. For TP-DSM20617, only the genomic region corresponding to the lysogeny module of TP-J34, bearing the *ltp* sequence, was amplified from genomic DNA by PCR and sequenced.

In this section, we will address features TP-J34 and TP-778L genomes have in common, before we present in more detail those data, which are specific for the four phages and distinguish them from other *S. thermophilus* phages. TP-J34 and TP-778L DNAs share the same typical organization of functional modules characteristic for temperate *S. thermophilus* phages. Starting with the gene encoding the integrase, the order is: lysogeny module followed by modules for replication, DNA packaging, head morphogenesis, tail morphogenesis, lysis and finally lysogenic conversion (**Figure 3A**). While the lysogeny modules are transcribed from right to left, transcription of all other genes is from left to right. In none of the two genomes tRNA genes were detected. Sequences identical or highly similar to CRISPR spacer sequences in *S. thermophilus* strains were found in both genomes (**Table 2**). Their positions are indicated in **Figure 3A**. Orientations of the sequences are such that they correspond with the directions of transcription. Both phage genomes share with some other *S. thermophilus* phage genomes a site of a potential -1 translational frame-shift (Xu et al., 2004), which fuses *orf41* with *orf42* (TP-J34: bp 22942–23087) and *orf38* with *orf39* (TP-778L: bp 22560–22705), the two *orf*s in front of the gene encoding the tape measure protein (TMP). This frame-shift is known to result in formation of the tail assembly chaperone (Xu et al., 2013). TP-J34 has been shown to be a *pac*-type phage (Neve et al., 2003). By the same experimental approach, namely showing that minor DNA restriction bands were not affected by heat treatment of digested DNA, TP-778L was shown to be a *pac*-type phage as well. This corresponds with the rather high similarity seen between both large terminase units (**Figure 3A**).

We compared the nucleotide sequence of TP-J34 with those of other *S. thermophilus* phages, for which complete genomes were available: O1205 (Stanley et al., 1997), Sfi21 and Sfi19 (Desiere et al., 1998), Sfi11 (Lucchini et al., 1999), 7201 (Stanley et al., 2000), DT1 (Tremblay and Moineau, 1999), 2972 (Levesque et al., 2005), 858 (Deveau et al., 2008), ALQ13.2, Abc2 (Guglielmotti et al., 2009), and 5093 (Mills et al., 2011). The alignments by DotPlot analysis are shown in **Figure 3B**. It appears that virulent phage Sfi11 and temperate phage TP-778 and O1205 are the most closely related to TP-J34. This is further reflected by the large number of putative gene products of these phages sharing highest homologies with those of TP-J34 (see **Table 3**).

TP-J34 DNA

The nucleotide sequence was determined for DNA isolated from purified phage particles obtained by mitomycin C treatment of lysogenic *S. thermophilus* J34, as described before (Neve et al., 1998, 2003). TP-J34 DNA consists of 45,606 bp, and thus it is the largest of the *S. thermophilus* phage DNAs sequenced so far (http://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?opt=virus&taxid=10699). It has a G+C content of 38.8%, which is similar to the 39% of its host (Bolotin et al., 2004). The sequence is accessible under NC_020197. Numbering of the TP-J34

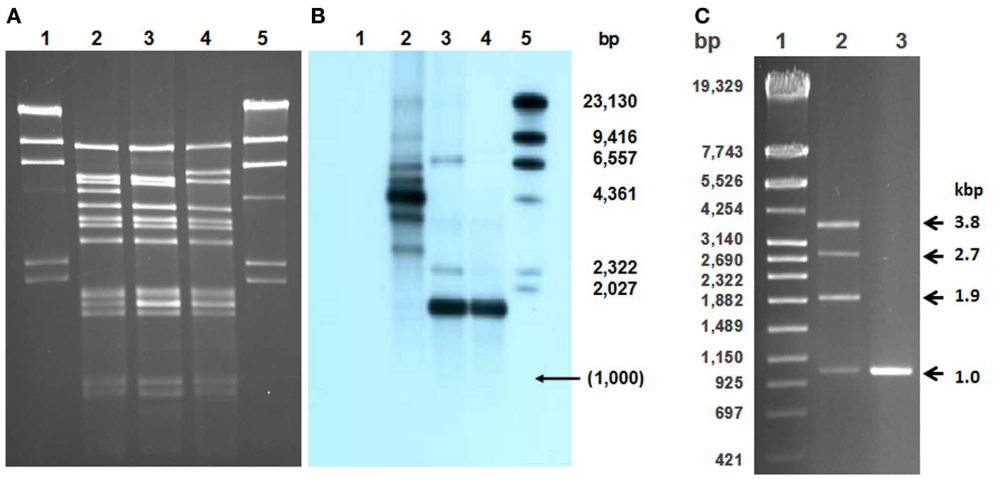

FIGURE 1 | Comparison of TP-J34, TP-J34L, and TP-EW genomic DNAs. Agarose gel **(A)** and corresponding Southern blot **(B)** of HindIII-cleaved DNAs of TP-J34 (lane 2), TP-J34L (lane 3), and TP-EW (lane 4) hybridized with DIG-labeled 1 kb probe generated from 1.7 kb HindIII fragment of TP-J34L. Lanes 1 and 5: unlabeled and Dig-labeled λ-DNA, respectively. Sizes of restriction fragments of λ-DNA are shown in the right margin. Agarose gel **(C)** of PCR-products generated from TP-J34 (lane 2) and TP-J34L (lane 3) DNA with primer pair D8+ und D12+. Lane 1: DNA molecular weight marker IV (Roche Diagnostics GmbH, Mannheim, Germany), sizes are indicated in the left margin. Sizes of PCR products are shown in the right margin.

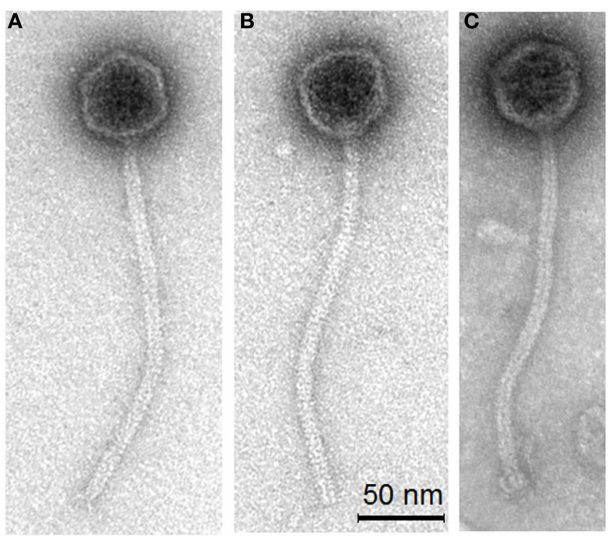

FIGURE 2 | Transmission electron micrographs of *S. thermophilus* phages TP-778L (A) propagated lytically on the prophage-cured derivative strain J34-2, phage TP-EW (B) and TP-DSM60217 (C) induced by mitomycin C from lysogenic *S. thermophilus* host strains EW and DSM20167, respectively.

sequence starts with the last nucleotide of the stop codon of the *int* gene.

Sixty *orf*s were predicted by the Artemis programme (Rutherford et al., 2000), all of which were considered as protein-encoding genes (**Table 3**) with protein sizes varying between 46 (*orf9*) and 1647 amino acids (*orf48*). The predominant start codon appears to be AUG (57 out of 60); one UUG (*orf23*), one AUU (*orf28*), and one CUG (*orf55*) were additionally predicted as start codons. AUU is a very unusual start codon (Blattner et al., 1997) normally coding for isoleucine. By repeated sequencing of PCR products generated with primers terS-F and terS-R using TP-J34 and TP-EW DNA, respectively, as templates, we excluded sequencing errors in this genomic region.

We have previously shown that upon induction of prophage TP-J34, mostly defective particles were released from the lysed host cells, and we have attributed the defect to a repeat region within *orf48* encoding the receptor binding protein (Neve et al., 2003). TP-J34L, an isolate forming clear plaques has been shown to have suffered a deletion of ca. 2.7 kb within the 4.4 kb HindIII fragment, thus reducing its size to 1.7 kb (Neve et al., 2003). In a Southern blot with HindIII-cleaved DNAs using a 1.0 kb PCR product (internal to the 1.7 kb HindIII fragment, obtained with primer pair D8/D12) of TP-J34L DNA as a probe, TP-J34 DNA extracted from lysates obtained by prophage induction yielded a major hybridization signal with the 4.4 kb fragment (**Figures 1A,B**). Two smaller signals at 3.5 and 2.6 kb were seen, indicating that the DNA was heterogeneous with respect to the 4.4 kb fragment, with 0.9 kb either one or two times deleted. As expected, TP-J34L DNA yielded a major signal at 1.7 kb. To confirm these results, the respective DNA regions of a TP-J34 lysate obtained by induction of the prophage and a TP-J34L lysate obtained by lytic propagation, were amplified by PCR, using primers D8+ and D12+ targeting sequences within the 4.4 kb HindIII fragment of TP-J34 but located outside of the repeat sequences. As expected, TP-J34L DNA gave rise to only one PCR product of ca. 1 kb. In case of the TP-J34 lysate, however, the DNA extracted yielded four products of ca. 1.0, 1.9, 2.8, and 3.7 kb (**Figure 1C**). This confirmed that TP-J34 DNA obtained by induction of the prophage was apparently heterogeneous with respect to the 4.4/1.7 kb HindIII fragment.

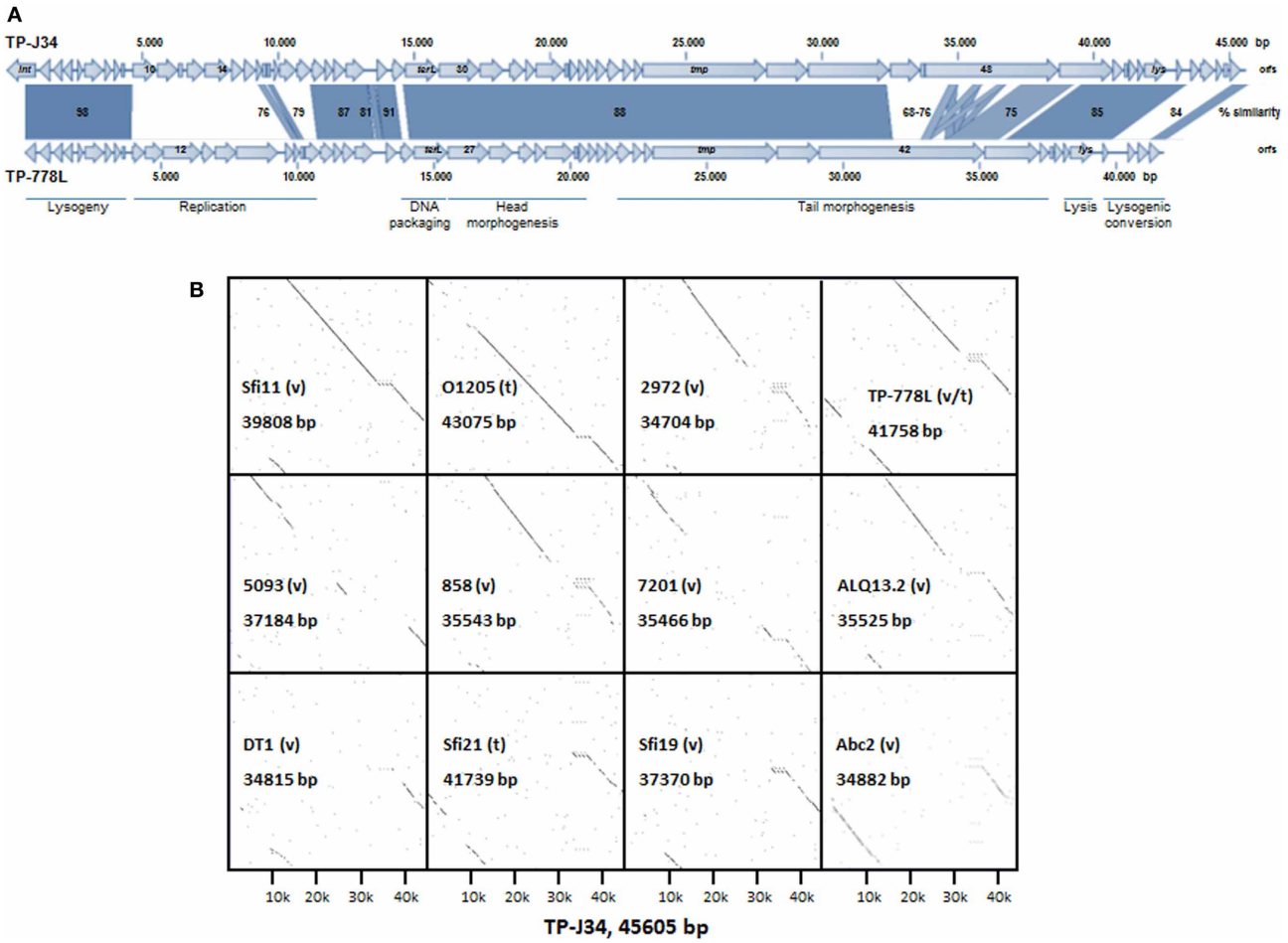

FIGURE 3 | (A) Alignment of gene maps and functional gene regions of TP-J34 and TP-778L. On the genetic maps, genes, and direction of transcription are indicted by arrows (very small genes are shown as boxes, the directions of transcription correspond to adjacent genes). Numbers or gene abbreviations refer to *orf*s or genes as listed in **Tables 3**, **4**. A scale indicating nucleotide positions is shown above the TP-J34 map. Approximate positions of functional regions (modules) are indicated by horizontal bars below the TP-778L map. Positions of CRISPR spacer sequences are indicated by dots above and below the maps of TP-J34 and TP-778L, respectively. **(B)** Dot plots of the TP-J34 nucleotide sequence compared to those of other *S. thermophilus* phages, including TP-778L. The horizontal line of each dot plot represents the 45,605 bp of TP-J34 DNA, whereas the vertical lines represent the numbers of bp for each phage, as indicated within each dot plot. Temperate (t) and virulent (v) phages are indicated.

Inspection of the TP-J34 genome sequence in this region revealed a 912 bp repeat structure within *orf48* (**Figure 4**), located between genome positions 34,630 and 37,367. The triplicated sequence (3 × 912 bp) was found to be entirely in frame with the coding sequence of *orf48* encoding the putative host specificity protein. Theoretically, a gene product should be produced, which—according to the defective morphology of TP-J34—should be either inactive in the tail assembly process or physically unstable. We like to point out that when the TP-J34 prophage was induced and the resulting lysate was inspected by transmission electron microscopy after fractionation in a CsCl gradient, no tail structures were detected anywhere in the gradient (Neve et al., 2003).

To genetically prove that the defect in *orf48* was responsible for the tail assembly defect, we used the lysate obtained by induction of the TP-J34 prophage, which contained mostly defective particles, for re-lysogenization of prophage-cured *S. thermophilus* J34-6. From 11 lysogens obtained, chromosomal DNA was isolated, restricted with HindIII and subjected to Southern blotting using the 1.0 kb PCR product of TP-J34L DNA as probe. Of the 11 strains, seven showed a hybridization signal at 1.7 kb, three a signal at 2.6 kb and one a strong signal at 1.7 and a weaker signal at 2.6 kb. Genomic DNA isolated from lysogenic *S. thermophilus* J34 yielded three signals at 2.6, 3.5, and 4.4 kb (**Figure 5**). Of two of the re-lysogenized strains, J34-6-RL2 (signal at 2.6 kb) and J34-6-RL4 (signal at 1.7 kb), prophage were induced with mitomycin C. The lysates obtained were subjected to electron microscopy and compared with lysates obtained by prophage induction of *S. thermophilus* J34 and by lytic propagation of TP-J34L. The vast majority of phage particles of TP-J34 and TP-J34-6-RL2 were defective, whereas about half of the TP-J34L and TP-J34-6-RL4 looked morphologically intact, when analyzed in the electron

Table 2 | CRISPR spacer sequences present in genomes of TP-J34 and TP-778L[a].

Sequence ID/*S. thermophilus* strain phage	Spacer sequence[b]	Identity	*E*-value
TP-J34			
NC_008532_5_4 /LMD-9	agagtacaatattgtcctcattggagacac 5882 5911	1	7e-07
NC_008532_4_3 /LMD-9	catcataggcggaactggtaggatgtacac 44252 44281	1	7e-07
NC_006449_1_31 NC_006449_1_5 /CNRZ1066	gttggcaatgcaaacaacctttatgaaccg 40182 40211	1	7e-07
NC_017563_1_29 /NDO3	gaaagaatcggtcttctagatggattccaa 5245 5274	0.97	1e-04
NC_006449_1_6 /CNRZ1066	aaaggtggaacgttatcgcaaggaaataaa 33041 33070	0.97	1e-04
NC_006449_1_41 /CNRZ1066	atttgaaaaatgcacaacagcgtttgata 38388 38416	0.97	4e-04
TP-778L			
NC_017563_3_3 /ND03	cggacagcgataaatacactctatacagaga 12541 12571	1	2e-07
NC_017927_3_4 /MN-ZLW-002	attgacctattcaatgtatgggtcacgtaa 38358 38387	1	7e-07
NC_008532_2_3 /LMD-9	agtaatgatggtcggttatttttcagacat 36793 36822	0.97	1e-04
NC_006448_1_17 /LMG 18311	cattaaatcgcttgaagcagacattgaagc 4072 4101	0.97	1e-04
NC_008532_2_16 /LMD-9	aacagttactattaatcacgattcc 35406 35430	1	4e-04

[a] *Only sequences with E-values < 0.001 are shown.*

[b] *The phage sequences are shown with positions of first and last nucleotide.*

microscope. When measuring plaque formation, phage lysates of TP-J34L and TP-J34-6-RL4 each yielded ca. 10^8 pfu/ml, while TP-J34 and TP-J34-6-RL-2 each yielded ca. 10^5 pfu/ml. It thus appears that even an insertion of one 912 bp repeat is sufficient for inactivation of the tail assembly function of *orf48* gene product.

TP-778

The nucleotide sequence was determined for DNA isolated from CsCl-purified TP-778L, lytically propagated on *S. thermophilus* B106, as described in Materials and Methods. TP-778L DNA consists of 41,757 bp. It has a G+C content of 39%, which is identical to the 39% of its host (Bolotin et al., 2004). The sequence is accessible under NC_022776. Numbering of the TP-J34 sequence starts with the last nucleotide of the stop codon of the *int* gene. Of the 52 *orf*s predicted by the Artemis programme (Rutherford et al., 2000), all were considered as protein-encoding genes (**Table 4**) with protein sizes varying between 46 (*orf9*) and 2020 amino acids (*orf42*). The predominant start codon appears to be AUG (49 out of 52). Of the residual three, two appear to be GUG (*orf*s *16* and *19*) and one UUG (*orf43*).

S. thermophilus SK778 could not be cured of its prophage. To find a host for lytic propagation, a set of 16 non-lysogenic *S. thermophilus* wild-type strains were tested for sensitivity to TP-778L. Only *S. thermophilus* strain B106, a host strain for propagation of temperate phage 7201 (Proux et al., 2002) which had been kindly provided by the University of Cork, Ireland, was found to allow plaque formation of TP-778L. Phage TP-778L was isolated as a plaque-purified, lytically propagated isolate. Its DNA sequence revealed that only a truncated integrase gene was present. Therefore, both host DNA regions flanking the prophage residing in the host genome were amplified by PCR and sequenced. Both flanking regions were found to be identical to *S. thermophilus* NDO3 DNA (Sun et al., 2011). The left region flanking the prophage's integrase gene contained a typical attachment site (Bruttin et al., 1997) overlapping with the 3′-end of the integrase gene of the prophage, which—in contrast to that of TP-778L—was complete. The right flanking region did not reveal an attachment site. Instead, a truncated integrase gene was seen, which showed high similarity to a phage remnant (Ventura et al., 2002). Comparison of the different integrase gene sequences indicated that excision of the prophage in case of TP-778L had occurred by recombination between the left complete and the right truncated integrase gene (**Figure 6**).

TP-EW

From an industrial yoghurt, we isolated lysogenic *S. thermophilus* strain EW carrying a prophage (called TP-EW). Upon induction with mitomycin C, a phage lysate of morphologically intact phage particles was obtained. Using a spot assay, TP-EW was shown to be able to productively infect *S. thermophilus* J34-6 (not shown). Restriction analysis with HindIII of DNA isolated from CsCl-purified phage particles revealed a pattern basically identical to TP-J34 DNA. Therefore, we consider this phage to be almost identical to TP-J34. However, two differences in the restriction pattern with respect to TP-J34 DNA were noticed (**Figure 1A**): the two fragments of TP-J34 of 5.0 and 4.4 kb were missing, instead, two new fragments of 1.7 and 6.0 kb were detected.

By Southern hybridization (**Figure 1B**) and DNA sequencing we could show that TP-EW DNA did not contain the 3 × 912 bp repeats found in the 4.4 kb fragment of TP-J34 DNA, but that it instead contained the fragment of 1.7 kb identical to the one of TP-J34L (**Figure 4**).

The second differing restriction fragment of ca. 6 kb, when analyzed by additional restriction hydrolyses (not shown), appeared to be altered within the region of the lysin gene (*orf54*) with respect to TP-J34. A PCR with primers LYSup and LYSdown (**Table 1**) showed that TP-J34 DNA yielded a product of ca. 1.0 kb, while that of TP-EW DNA was ca. 1 kb larger (not shown). DNA sequencing and comparison with the TP-J34 DNA sequence indicated that the lysin gene of TP-EW contained an insertion of 1016 bp. BlastX analysis of the inserted sequence revealed an open reading frame encoding a protein of 205 amino acids with high homology to homing endonucleases (Lambowitz, 1993), indicating that the inserted sequence

Table 3 | Features of phage TP-J34 *orf*s and putative functions of their products.

ORF (gene)	DNA frame	Start	Stop	Size (aa)	SD sequence AAGGAGGT[a]	Predicted function/best match BLASTp result	*E*-value	Match identity (%)	References, acc. no.
1 (*int*)	−1	1080	1	359	TTGGGGGAttaaataa**ATG**	Integrase/ *S. thermophilus* phage Sfi21, Integrase/ 359	0.0	100	Desiere et al., 1998
2 (*ltp*)	−3	1612	1184	142	ATGGAGGAaattttt**ATG**	Superinfection exclusion lipoprotein/ *Streptococcus parasanguinis*, prophage superinfection immunity protein 152	3e-42	51	-/ WP_003010598
3	−1	2061	1693	122	AAAGTGAGaatttt**ATG**	Putative metallo-proteinase/ *S. thermophilus* phage Sfi21, similar to cI-like repressor, metallo-proteinase motif	1e-53	82	/ Desiere et al., 1998
4 (*crh*)	−1	2433	2068	121	AAGGAGAAagat**ATG**	Putative CI-repressor/ *S. thermophilus* phage Sfi21, CI-like repressor	8e-21	55	/ Desiere et al., 1998
5 (*cro*)	+1	2602	2805	67	GAGGAGAAacaaa**ATG**	Putative Cro protein/ *S. thermophilus* phage 7201, Orf1, cro-like protein homolog	4e-28	91	/ Stanley et al., 2000
6 (*ant*)	+2	2858	3574	238	AAGGATAAtac**ATG**	Putative antirepressor/ *S. thermophilus* phage Abc2, antirepressor protein	2e-129	98	/ Guglielmotti et al., 2009
7	+1	3595	3876	93	ATAGGGGTtgaaaaagact**ATG**	-/ *S. thermophilus* phage Sfi21, Orf80	5e-47	98	/ Desiere et al., 1998
8	+3	3936	4199	87	AAGGAATTaaa**ATG**	-/ *S. thermophilus* phage Sfi21, Orf87	3e-44	100	/ Desiere et al., 1998
9	+2	4217	4357	46	GAGGAGAAacaaa**ATG**	-/ S. pyogenes phage315.5, hypothetical protein SpyM3_1347	4.4	41	/ Beres et al., 2002
10	+1	4630	5517	295	GGGTGAGTctaaa**ATG**	-/ *S. thermophilus* phage 5093, putative primosome component	1e-142	99	/ NC_012753
11	+2	5529	6311	260	AAAGGGGTtgact**ATG**	-/ *S. thermophilus* phage 5093, DnaC-like protein	5e-136	93	/ NC_012753
12	+3	6308	6490	60	CAAGAGGAtgatgct**ATG**	-/ *S. thermophilus* phage 5093, hypothetical protein	6e-27	100	/ NC_012753
13	+2	6615	7277	220	AAGGGAGAtaaa**ATG**	-/ *S. thermophilus* phage 5093, putative Erf protein	1e-122	98	NC_012753

(Continued)

Table 3 | Continued

ORF (gene)	DNA frame	Start	Stop	Size (aa)	SD sequence AAGGAGGT[a]	Predicted function/best match BLASTp result	*E*-value	Match identity (%)	References, acc. no.
14	+1	7279	8238	319	AAGGAGAActagc**ATG**	-/ *S. thermophilus* phage Abc2, hypothetical protein	7e-146	82	Guglielmotti et al., 2009
15	+1	8261	8710	149	CAGGAGAAaaaaac**ATG**	-/ *S. thermophilus* phage Abc2, single-stranded DNA binding protein	1e-73	90	Guglielmotti et al., 2009
16	+1	8719	9180	153	AAGGGAAAct**ATG**	-/ *S. thermophilus* phage Abc2, hypothetical protein	1e-82	97	Guglielmotti et al., 2009
17	+3	9177	9413	78	AAGGAGCTgga**ATG**	-/ *S. thermophilus* temperate phage O1205, hypothetical protein	3e-31	83	Stanley et al., 1997
18	+2	9404	9574	56	ATGGAGGAact**ATG**	-/ *S. thermophilus* phage Abc2, hypothetical protein	6e-19	85	Guglielmotti et al., 2009
19	+1	9571	9726	51	AAGGAGATtgattgaatt**ATG**	-/ *S. thermophilus* phage Sfi21, hypothetical protein	2e-17	87	Desiere et al., 1998
20	+3	9822	10028	68	AAAGAGGTaaattaa**ATG**	/ *Streptococcus pneumoniae*, hypothetical protein	6e-11	62	ZP_01829218
21	+3	10029	10670	213	AAAGAGGTggaatag**ATG**	/ *S. pyogenes* phage 2096.1, phage protein	8e-91	70	Beres et al., 2002
22	+1	10672	11217	181	TTGGAGAAaataaa**ATG**	/ *S. thermophilus* phage Sfi21, Orf178	5e-86	88	Lucchini et al., 1999
23	+3	11220	11732	170	AAAGAGGTgtaata**TTG**	/ *S. thermophilus* phage 858, DNA binding protein (170aa)	4e-80	86	Deveau et al., 2008
24	+1	11701	12018	105	AGGGAAGAtagtaa**ATG**	/ *S. thermophilus* phage Sfi18, gp99	1e-43	94	Lucchini et al., 1999
25	+2	12020	12463	147	GTAGAGGTaattaag**ATG**	/ *S. thermophilus* phage Sfi11, hypothetical protein	1e-64	99	Lucchini et al., 1999
26	+1	12469	13179	236	GCGTAGGAttc**ATG**	/ *S. thermophilus* phage 858, Orf46	3e-117	86	Deveau et al., 2008
27	+1	13615	14028	137	AGAGAGGTtagtaca**ATG**	/ *S. thermophilus* phage Sfi11, gp137, ArpU phage transcriptional regulator	5e-72	95	Lucchini et al., 1999

(Continued)

Table 3 | Continued

ORF (gene)	DNA frame	Start	Stop	Size (aa)	SD sequence AAGGAGGT[a]	Predicted function/best match BLASTp result	*E*-value	Match identity (%)	References, acc. no.
28 (*terS*)	+1	14177	14671	165	AAGGAGGTggatgt**ATT**	Putative terminase small subunit/ *S. thermophilus* phage Sfi11, gp172, putative terS product	2e-111	98	Lucchini et al., 1999
29 (*terL*)	+2	14658	15893	411	AAGGAGCTgtaaaca**ATG**	Putative terminase large subunit/ *S. thermophilus* phage Sfi11, gp411, putative terL product	0	98	Lucchini et al., 1999
30	+1	15899	17407	502	TAGGAGGaatg**ATG**	Putative portal protein/ *S. thermophilus* phage Sfi11, gp502, portal protein	0	99	Lucchini et al., 1999
31	+2	17404	18297	297	GAGAGGGTttatga**ATG**	/ *S. thermophilus* phage Sfi11, gp284, putative minor head protein; /	5e-144	92	Lucchini et al., 1999
32	+2	18486	19067	193	TAGGAGAAataa**ATG**	/ *S. thermophilus* phage Sfi11, gp193, putative scaffold protein	2e-105	99	Lucchini et al., 1999
33	+3	19087	19446	119	AAGGATTTtttaa**ATG**	/ *S. thermophilus* temperate phage O1205, Orf30, putative structural protein	6e-57	94	Stanley et al., 1997
34	+3	19465	20511	348	GAGGAGGAatattaaaac**ATG**	Putative major head protein/ *S. thermophilus* phage Sfi11, gp348, major head protein	0	97	Lucchini et al., 1999
35	+2	20523	20684	53	GAGGTGCTact**ATG**	/ *S. thermophilus* phage Sfi11, gp53	3e-22	100	Lucchini et al., 1999
36	+3	20696	21037	113	AGCGAGGTgtggc**ATG**	/ *S. thermophilus* temperate phage O1205, hypothetisches Protein	4e-57	96	Stanley et al., 1997
37	+2	21034	21348	104	GGTGAGGTgctatttct**ATG**	/ *S. thermophilus* phage Sfi11, gp104	6e-54	100	Lucchini et al., 1999
38	+2	21348	21692	114	AAGGTGGTtagata**ATG**	/ *S. thermophilus* phage Sfi11, gp114	7e-60	100	Lucchini et al., 1999
39	+1	21689	22075	128	TGGGATGAaac**ATG**	/ *S. thermophilus* phage Sfi11, gp128	3e-71	100	Lucchini et al., 1999
40	+3	22088	22594	168	TAGGAGGAaaaa**ATG**	Putative major tail protein/ *S. thermophilus* temperate phage O1205, Orf37, major tail protein	7e-90	99	Stanley et al., 1997

(Continued)

Table 3 | Continued

ORF (gene)	DNA frame	Start	Stop	Size (aa)	SD sequence AAGGAGGT[a]	Predicted function/best match BLASTp result	*E*-value	Match identity (%)	References, acc. no.
41	+2	22669	23022	117	TAGGAGTAaacaaacaa**ATG**	/ *S. thermophilus* phage Sfi11, gp117	2e-61	100	Lucchini et al., 1999
42	+2	23085	23402	105	TACGAGGAattaatcacgaatgct**ATG**	/ *S. thermophilus* phage Sfi11, gp105	1e-51	100	Lucchini et al., 1999
43 (*tmp*)	+2	23392	27945	1517	AGAGAGGGgcttgctag**ATG**	Putative tape measure protein/ *S. thermophilus* phage Sfi11, gp1510, putative minor tail protein	0	95	Lucchini et al., 1999
44	+2	27945	29483	512	TGAGAGGTctcaatta**ATG**	/ *S. thermophilus* phage Sfi11, gp512, putative minor tail protein	0	94	Lucchini et al., 1999
45	+1	29483	32485	1000	AAGGTGGAttta**ATG**	/ *S. thermophilus* phage Sfi11, gp1000, putative minor tail protein (Lysozyme and Chap domain)	0	97	Lucchini et al., 1999
46	+1	32501	33622	373	TAGGAGGAattaaat**ATG**	/ *S. thermophilus* phage Sfi11, gp373	0	98	Lucchini et al., 1999
47	+3	33622	33795	57	TGTGAGGTgaatcaata**ATG**	/ *S. thermophilus* phage Sfi11, gp57	7e-24	94	Lucchini et al., 1999
48	+1	33773	38716	1647	GCGGAGTTaagta**ATG**	Putative host specificity protein / *S. thermophilus* phage DT2, host specificity protein	0	72	Duplessis and Moineau, 2001
49	+2	38718	40727	669	TAGGAGAAgattaaa**ATG**	/ *S. thermophilus* phage Sfi11, gp669, putative minor structural protein	0	96	Lucchini et al., 1999
50	+1	40691	41092	133	AAAATGG**ATG**	/ *S. thermophilus* phage Sfi11, gp149	1e-59	76	Lucchini et al., 1999
51	+2	41112	41258	48	AAAGAGGAaaaagat**ATG**	/ *S. thermophilus* phage Sfi21, hypothetical protein	9e-12	75	Desiere et al., 1998
52	+2	41276	41599	107	AGGGATGTgtt**ATG**	/ *S. thermophilus* phage DT1, Orf23	3e-53	95	Lamothe et al., 2005
53 (*hol*)	+3	41605	41847	80	TGAGAGGAtaaagaca**ATG**	Putative holin/ *S. thermophilus* temperate phage O1205, putative holin	4e-35	93	Stanley et al., 1997

(Continued)

Table 3 | Continued

ORF (gene)	DNA frame	Start	Stop	Size (aa)	SD sequence AAGGAGGT[a]	Predicted function/best match BLASTp result	*E*-value	Match identity (%)	References, acc. no.
54 (*lys*)	+1	41849	42694	281	AGGAAGGAaaataat**ATG**	Putative lysin/ *S. thermophilus* phage S3B, putative lysin	7e-141	90	Foley et al., 2000
55	+2	42858	43256	132	AAGAAAAAcggctattgac**CTG**	/ *S. pneumoniae*, hypothetical protein (trans-membrane region)	5e-12	42	NZ_ABAA01000017
56	+1	43557	43892	111	AAAGAGGAaatgaa**ATG**	/ *S. thermophilus* phage Sfi19, gp111	8e-55	100	Desiere et al., 1998
57	+1	43914	44465	183	AAGGAGAAataaaaa**ATG**	/ *S. thermophilus* phage Sfi11, gp183	5e-104	100	Lucchini et al., 1999
58	+2	44491	44742	83	AACGAGGTgaaaaca**ATG**	/ *S. thermophilus* phage Sfi11, gp83	6e-40	100	Lucchini et al., 1999
59	+1	44768	44947	59	AAGCTTTAactgat**ATG**	/ *S. thermophilus* phage 5093, hypothetical protein	1e-25	93	NC_012753
60	+1	45006	45428	140	GAGGAAGTaaatgaa**ATG**	/ *S. thermophilus* phage 5093, hypothetical protein	4e-63	85	NC_012753

[a] *da Silva Oliveira et al., 2004.*

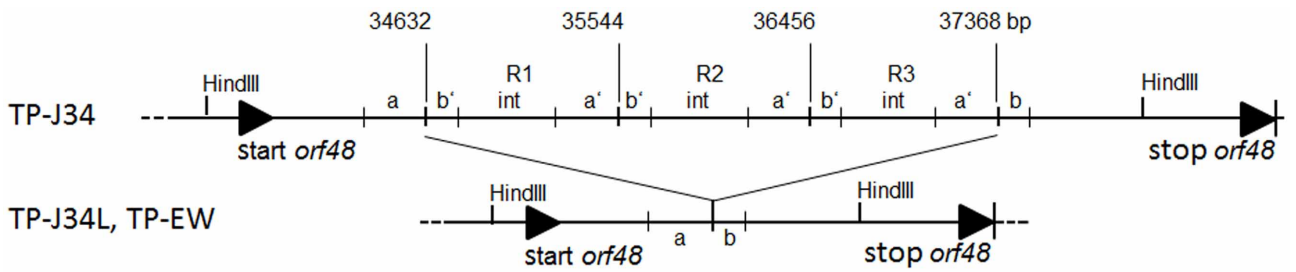

FIGURE 4 | Comparison of the genetic structure of the TP-J34 DNA region containing the triple repeat sequences R1–R3 with that of TP-J34L and TP-EW, respectively. The bp numbers indicate the first bp of a repeat. "a" and "b" denote the regions with similarities to sequences within the repeats (marked as "a" and "b"). Sequences exclusively found within the three repeats are indicated as "int." HindIII restriction sites flanking the 4.4 and 1.7 kb fragment of TP-J34 and TP-J34L/TP-EW, respectively are shown. Gene 48 start and stop are marked by solid triangles.

is a group I intron. Such introns have frequently been found in *S. thermophilus* phages to be located within the lysin gene (Foley et al., 2000). Comparison of the putative splice sites indicated high homology between *S. thermophilus* phages containing an intron in that position (**Figure 7**). Comparison of the DNA sequences flanking the insertion site of the intron with TP-J34 DNA sequence of that region revealed many deviations from TP-J34 sequence in the close vicinity, while the DNA sequences of TP-EW and TP-J34 were identical when they were more than a few hundred nucleotides apart from the insertion site.

Finally, for sequencing the ltp_{TP-EW} gene, we amplified a DNA region comprising the *ltp* gene plus the flanking regions by means of primers targeting sequences of TP-J34 genes *int* and *orf3*, respectively. The ca. 900 bp of nucleotide sequence obtained were 100% identical to those of TP-J34.

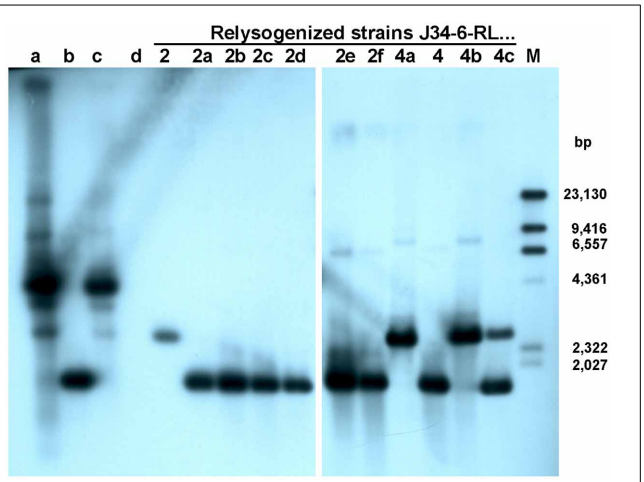

FIGURE 5 | Southern blot with DIG-labeled 1 kb probe of HindIII-cleaved phage and chromosomal DNA of eleven *S. thermophilus* strains relysogenized with TP-J34. Lane a: TP-J34; lane b: TP-J34L; lane c: J34; lane d: J34-6 (no prophage, negative control); lane M: DIG-labeled, HindIII-cleaved λ DNA. Other lanes (from left to right): J34-RL2; J34-6-RL2a; J34-6-RL2b; J34-6-RL2c; J34-6-RL2d; J34-6-RL2e; J34-6-RL2f; J34-6-RL4a; J34-6-RL4; J34-6-RL4b; J34-6-RL4c. The sizes of the λ DNA bands are indicated in the right margin.

TP-DSM20617

S. thermophilus DSM20617 was obtained from the German type culture collection. It had been included in a screening for lysogenic *S. thermophilus* strains carrying *ltp*-expressing prophages (Sun, 2002). The DNA region of lysogenic strain *S. thermophilus* DSM20617 comprising *orf1* (integrase) through *orf6* (antirepressor) and defined by primers primer4 (left) and Mz12.R (right) was sequenced by primer walking. The sequence of ca. 3.7 kb was more than 99% identical to that of prophage TP-778 residing in *S. thermophilus* SK778. Only one base within orf1 (*int*), one base within orf2 (*ltp*), and two bases within orf 5 (*ant*) turned out to be different. Restriction analyses of DNA isolated from the phage lysate obtained by induction of the prophage did not reveal any similarities to restriction patterns of DNA isolated from TP-J34L and TP-778L, respectively (Figure S1A). Also, comparison of the HindIII and EcoRI patterns of TP-DSM20617 DNA with *in silico* generated patterns of 11 *S. thermophilus* phage genomes did not reveal any similarities (Figures S1B,C).

STRUCTURAL AND FUNCTIONAL ASPECTS OF *ltp* GENES AND PRODUCTS

We compared the *ltp* gene products of the four phages (**Figure 8**). While $Ltp_{TP\text{-}J34}$ and $Ltp_{TP\text{-}EW}$ were identical, $Ltp_{TP\text{-}778}$ and $Ltp_{TP\text{-}DSM20617}$ differed in just one amino acid. However, both amino acid sequences of the mature proteins differed from that of mature $Ltp_{TP\text{-}J34}$ in eight ($Ltp_{TP\text{-}778}$) and nine ($Ltp_{TP\text{-}DSM20617}$) positions, respectively. Most deviations were conservative substitutions (e.g., D vs. E) and were found within the first of the two repeat regions of the Ltp protein. We like to point out that in 2014 two protein sequences became available, which match the $Ltp_{TP\text{-}DSM20617}$ sequence by 100%. One is from *S. thermophilus* prophage 20617 (Acc. no. CDG57923) and the other is from *S. thermophilus* M17PTZA496 (Acc. no. ETW90609).

To functionally compare $Ltp_{TP\text{-}778}$ with $Ltp_{TP\text{-}J34}$, we cloned $ltp_{TP\text{-}778}$ in pMG36e, yielding plasmid pYAL1-3, exactly as $ltp_{TP\text{-}J34}$ had been cloned to yield pXMS2 (Sun et al., 2006). After transformation of pYAL1-3 into *L. lactis* Bu2-60, the plating efficiencies of three lactococcal phages, which had already been tested against $Ltp_{TP\text{-}J34}$ (Sun et al., 2006), were determined. Activity of $Ltp_{TP\text{-}778}$ proved to be distinct from that of $Ltp_{TP\text{-}J34}$: instead of strong inhibition of P008 as seen by $Ltp_{TP\text{-}J34}$ almost no inhibition by $Ltp_{TP\text{-}778}$ was recorded. Infection of phage P001, on the other hand was significantly impaired by $Ltp_{TP\text{-}778}$, while $Ltp_{TP\text{-}J34}$ did show almost no activity against P001 (**Table 5**).

To further broaden our knowledge on Ltp activity, we tested 11 additional virulent lactococcal phages by a semi-quantitative spot assay (**Table 6**). Based on their morphologies as determined by electron microscopy, these phages had been assigned to the three different species c2, 936, and P335, represented by the three phages described in **Table 5**. P008, P001, and P335 were included as controls in the assay. In general, the control phages were inhibited by the different Ltp proteins to extends similar as those presented in **Table 5**. However, the phages assigned to one species did not show homogeneous behavior. While two phages of the c2-species were not inhibited by $Ltp_{TP\text{-}J34}$, three were strongly inhibited by this protein. On the other hand, one phage of this group was not inhibited by $Ltp_{TP\text{-}778}$, while all other phages of this group were significantly inhibited. Such non-homogeneous behavior was also seen for the phages from the two other species. One should bear in mind that assignment to the species has to be considered preliminary. However, all phages assigned to the two species 936 and P335were inhibited to below detection level by the secreted, non-lipoprotein derivative UsLtp1, as has been described before for the three control phages (Bebeacua et al., 2013).

DISCUSSION

Our screening for Ltp-expressing prophages in *S. thermophilus* yielded just four different phages, three of which (TP-J34, TP-EW, TP-778) can be assigned to the Sfi11 sub-species species of *S. thermophilus* phages (Proux et al., 2002; Quiberoni et al., 2010), since they are *pac*-type phages and their genome sequences show high similarities to phages Sfi11 and O1205. The fourth phage, TP-DSM20617 cannot be classified due to lack of information on its genome. The three phages, TP-J34 and TP-EW on one hand and TP-778 on the other, appear to represent two different lines within the Sfi11 sub-species, with the major difference between the two types being lack of homology between the genes within the "replication" module. Other minor differences are seen within the modules of "DNA-packaging," "tail morphogenesis," and "lysogenic conversion." The exchange of entire functional modules appears to be the general mechanism of recombination between bacteriophages (Lucchini et al., 1998). Such exchange is easily accomplished without impairing functionality of the phage, especially when interaction with proteins of other modules does not occur. This is the case with the proteins of the "replication" as well as the "lysogenic conversion" module. The "DNA packaging" module consists of two proteins only,

Table 4 | Features of phage TP-778L *orf*s and putative functions of their products.

Orf (gene)	DNA-frame	Start	Stop	Size (aa)	SD sequence AAGGAGGT[a]	Predicted function/best match BLASTp result	*E*-value	Match identity (%)	References, acc. no.
1 (int)	−3	420	1	139	TTGGGGGAttaaataa**ATG**	Putative integrase/ *Streptococcus thermophilus* phage *Sfi*21, integrase / 359	2e-90	99	Desiere et al., 1998, NP_049990
2 (ltp)	−1	952	524	142	TGGTAGGAaattt**ATG**	Putative superinfection exclusion lipoprotein/ *Streptococcus thermophilus* phage TP-J34/ 142	3e-79	93	Neve et al., 1998, AAC03455
3	−2	1400	1032	122	AAGGAAAAgtgagaattt**ATG**	Putative metallo- proteinase motif/ *Streptococcus thermophilus* phage *Sfi*21, cI-like repressor/ 122	4e-81	96	Desiere et al., 1998, NP_049992
4 (crh)	−2	1772	1407	121	AAGGAGAAagat**ATG**	Putative CI- repressor/ *Streptococcus thermophilus* phage TP-J34, putative cI-repressor homolog/ 121	1e-80	100	Neve et al., 1998, AAC03457
5 (cro)	+3	1941	2144	67	GAGGAGAAacaaa**ATG**	Putative Cro protein/ *Streptococcus thermophilus* phage TP-J34, Cro-like regulatory protein/ 67	3e-41	99	Neve et al., 1998, AAC03458
6 (ant)	+1	2197	2913	238	AGAAAGGAtaatac**ATG**	Putative antirepressor/ *Streptococcus thermophilus* phage TP-J34, P1-antirepressor homolog / 238	2e-175	99	Neve et al., 1998, AAC03459
7	+3	2934	3215	93	ATAGGGGTtgaaaaagact**ATG**	-/ *Streptococcus thermophilus* phage TP-J34, hypothetical protein/ 93	1e-61	100	Neve et al., 1998, AAC03460
8	+2	3275	3538	87	AAGGAATTaaa**ATG**	-/ *Streptococcus thermophilus* phage *Sfi*21 Orf87, hypothetical protein *Sfi*21p33/ 87	6e-57	100	Desiere et al., 1998, NP_597801
9	+1	3556	3693	46	AAAGAGGAgaaacaaa**ATG**	-/ *Streptococcus thermophilus* phage TP-J34, hypothetical protein/ 46	4e-26	100	This study
10	+2	3932	4405	157	AAGGAGTAtaccataaaat**ATG**	-/ *Streptococcus thermophilus* phage ALQ13.2, hypothetical protein/ 157	4e-88	84	Guglielmotti et al., 2009, YP_003344879

(Continued)

Table 4 | Continued

Orf (gene)	DNA-frame	Start	Stop	Size (aa)	SD sequence AAGGAGGT[a]	Predicted function/best match BLASTp result	*E*-value	Match identity (%)	References, acc. no.
11	+1	4402	5103	233	AAGGAGAAaccttaacataag**ATG**	-/ *Streptococcus thermophiles* phage, putative replication initiation protein/ 233	3e-168	99	Levesque et al., 2005, YP_238517
12	+2	5060	6472	470	AAAGGGGTgtaaggtag**ATG**	-/ *Streptococcus thermophilus* phage 858 Orf 37, putative helicase/ 470	0.0	99	Deveau et al., 2008, YP_001686831
13	+2	6479	6952	157	TTGGAGATaaaaaaac**ATG**	-/ *Streptococcus thermophilus* phage 858 Orf 38/ 157	3e-108	97	Deveau et al., 2008, YP_001686832
14	+3	6957	7772	271	TTTGCCATtctaagact**ATG**	-/ *Streptococcus thermophilus* phage 858 Orf 39, primase-polymerase domain/ 271	0.0	99	Deveau et al., 2008, YP_001686833
15	+1	7741	9297	518	AAGGAGTTagatactaaac**ATG**	Putative primase/ *Streptococcus* phage YMC-2011, putative primase / 519	0.0	92	Geng et al., 2011, YP_006561246
16	+1	9541	9861	106	AGAAAGGTaaattttaa**GTG**	-/ *Streptococcus thermophilus* phage 858 Orf 41, VRR_NUC domain/ 106	1e-65	92	Deveau et al., 2008, YP_001686835
17	+2	9845	10081	78	AAGGAAGCtttggatatagtaa**ATG**	-/ *Streptococcus thermophilus* phage Abc2, hypothetical protein/ 78	7e-42	87	Guglielmotti et al., 2009, YP_003347446
18	+3	10098	10253	51	AAGATGGTagagtt**ATG**	-/ *Streptococcus thermophilus* phage Sfi19 Orf 51; hypothetical protein Sfi19p40/ 51	1e-23	84	Desiere et al., 1998, NP_049960
19	+3	10254	10835	193	GAGGTGGAataa**GTG**	-/ *Streptococcus thermophilus* phage Abc2, hypothetical protein/ 166	9e-58	69	Guglielmotti et al., 2009, YP_003347451
20	+3	10836	11348	170	GAAGAGGTtgaataa**ATG**	Putative DNA-binding protein/ *Streptococcus thermophilus* phage 5093, DNA binding protein, HTH_XRE/ 170	6e-111	91	Mills et al., 2011, YP_002925093

(Continued)

Table 4 | Continued

Orf (gene)	DNA-frame	Start	Stop	Size (aa)	SD sequence AAGGAGGT[a]	Predicted function/best match BLASTp result	*E*-value	Match identity (%)	References, acc. no.
21	+1	11317	11634	105	AGGGAAGAtagtaa**ATG**	-/ *Streptococcus thermophilus* phage TP-J34, hypothetical protein/ 105	1e-65	95	This study
22	+2	11636	12079	147	GTAGAGGTaattaag**ATG**	-/ *Streptococcus thermophilus* phage TP-J34, hypothetical / 147	2e-103	99	This study
23	+1	12085	12795	236	GTGGGGGCgtaggattc**ATG**	/ *Streptococcus thermophilus* phage 7201 Orf 18/ 235	7e-161	94	Stanley et al., 2000, NP_038319
24	+2	13232	13645	137	AGAGAGGGcagaaaa**ATG**	Putative transcriptional regulator/ *Streptococcus thermophilus* phage TP-J34 Orf27, transcriptional regulator ArpU family/ 137	2e-94	99	This study
25 (terS)	+2	13766	14278	170	TTTGAGTTgtctttttttgattatgaa**ATG**	Putative terminase small subunit/ *Streptococcus thermophilus* phage 2972, terminase small subunit/ 150	7e-85	86	Levesque et al., 2005, YP_001686797
26 (terL)	+3	14265	15500	411	AAGGAGCTgttagcg**ATG**	Putative terminase large subunit/ *Streptococcus thermophilus* phage TP-J34 Orf29, putative terminase large subunit / 411	0.0	97	This study
27	+2	15506	17014	502	TAGGAGGAatg**ATG**	Putative portal protein/ *Streptococcus thermophilus* phage 858 orf6, putative portal protein/ 502	0.0	97	Deveau et al., 2008, YP_001686800
28	+1	17011	17904	297	GAGAGGGTtatga**ATG**	Putative head protein/ *Streptococcus thermophilus* phage 2972, head protein/ 297	0.0	96	Levesque et al., 2005, YP_238489
29	+3	18096	18677	193	TAGGAGAAcaaa**ATG**	Putative scaffold protein/ *Streptococcus thermophilus* phage 2972, scaffold protein/ 193	7e-130	96	Levesque et al., 2005, YP_238490
30	+1	18697	19056	119	AAGGAAATtttaa**ATG**	Putative head protein/ *Streptococcus thermophilus* phage 2972, head protein/ 119	6e-75	97	Levesque et al., 2005, YP_238491

(Continued)

Table 4 | Continued

Orf (gene)	DNA-frame	Start	Stop	Size (aa)	SD sequence AAGGAGGT[a]	Predicted function/best match BLASTp result	*E*-value	Match identity (%)	References, acc. no.
31	+1	19075	20121	348	GAGGAGGAacattaaaac**ATG**	Putative capsid protein/ *Streptococcus thermophilus* phage ALQ13, capsid/ 348	0.0	98	Guglielmotti et al., 2009, YP_003344853
32	+3	20133	20294	53	TAAGAGGTactgat**ATG**	-/ *Streptococcus thermophilus* phage ALQ13, hypothetical protein/ 53	5e-19	98	Guglielmotti et al., 2009, YP_003344854
33	+2	20309	20647	112	AGTGAGGTatggcgtg**ATG**	-/ *Streptococcus thermophilus* phage 01205 Orf 33, hypothetical protein/ 122	3e-70	94	Stanley et al., 1997, NP_695111
34	+1	20644	20958	104	GGTgaggtgctatttct**ATG**	-/ *Streptococcus thermophilus* phage 2972, hypothetical protein / 104	7e-62	94	Levesque et al., 2005, YP_238495
35	+2	20960	21304	114	AAGGTGaTgaaataac**ATG**	-/ *Streptococcus thermophilus* phage Sfi11 Orf 114, hypothetical protein/ 114	4e-72	94	Lucchini et al., 1999, NP_056684
36	+1	21289	21687	132	GAAGAGATggcgaa**ATG**	-/ *Streptococcus thermophilus* phage ALQ13, hypothetical protein/ 128	1e-84	95	Guglielmotti et al., 2009, YP_003344858
37	+2	21701	22210	169	AATTAGGAGGAaaaa**ATG**	Putative tail protein/ *Streptococcus thermophilus* phage 2972, tail protein/ 169	9e-115	98	Levesque et al., 2005, YP_238498
38	+3	22287	22640	117	TAGGAGTAaacaaaca**ATG**	-/ *Streptococcus thermophilus* phage 2972, hypothetical protein / 117	6e-78	99	Levesque et al., 2005, YP_238499
39	+2	22703	23020	105	GAGGAGTTaatcactaatgcc**ATG**	-/ *Streptococcus thermophilus* phage 2972, hypothetical protein / 105	2e-65	99	Levesque et al., 2005, YP_238500
40 (tmp)	+3	23010	27563	1517	AGAGAGGGgcttgctag**ATG**	Putative tape measure protein/ *Streptococcus thermophilus* phage O1205, putative tail protein/ 1517	0.0	90	Stanley et al., 2000 NP_695118

(Continued)

Table 4 | Continued

Orf (gene)	DNA-frame	Start	Stop	Size (aa)	SD sequence AAGGAGGT[a]	Predicted function/best match BLASTp result	*E*-value	Match identity (%)	References, acc. no.
41	+2	27563	29101	512	TGCGAGGTctaaatta**ATG**	Putative tail protein/ *Streptococcus thermophilus* phage Sfi11, putative minor structural protein/ 511	0.0	89	Lucchini et al., 1999, NP_056690
42	+1	29101	35163	2020	AAGGTGGAttta**ATG**	Putative host specificity protein/ *Streptococcus thermophilus* phage 858 Orf21,prophage tail protein / 1006	0.0	88	Deveau et al., 2008, YP_001686815
43	+1	35164	37185	673	TAGGAGGTttttaa**TTG**	Putative tail protein/ *Streptococcus thermophilus* phage 858 Orf22/ 673	0.0	89	Deveau et al., 2008, YP_001686816
44	+1	37201	37548	115	AAGAAGGAaaattc**ATG**	-/ *Streptococcus thermophilus* phage TP-J34, hypothetical protein/ 133	6e-73	96	This study
45	+2	37568	37714	48	AAAGAGGAaaaagat**ATG**	-/ *Streptococcus thermophilus* phage TP-J34, hypothetical protein/ 48	2e-24	100	This study
46	+1	37732	38055	107	TAGGAGGGatgtgtt**ATG**	-/ *Streptococcus thermophilus* phage TP-J34, hypothetical protein/ 107	2e-71	100	This study
47 (hol)	+3	38064	38306	80	TGAGAGGAtaaataacaat**ATG**	Putative holin/ *Streptococcus thermophilus* phage Abc2, holin/ 80	7e-44	93	Guglielmotti et al., 2009, YP_003347430
48 (lys)	+1	38308	39153	281	AAGGAAGGaaaatagt**ATG**	Putative lysin/ *Streptococcus thermophilus* phage TP-J34, putative lysin/ 281	8e-181	90	This study
49	+1	39499	39720	73	AAGATTGAaaacaaactagacgac**ATG**	-/ *Streptococcus thermophilus* phage TP-J34/ 73	5e-46	100	Neve et al., 1998, AAC03448
50	+3	40419	40715	98	AGAGAGGTaaaaagaa**ATG**	-/ *Streptococcus* sp. F0441, hypothetical protein/ 101	2e-38	66	WP_009730541

(Continued)

Table 4 | Continued

Orf (gene)	DNA-frame	Start	Stop	Size (aa)	SD sequence AAGGAGGT[a]	Predicted function/best match BLASTp result	*E*-value	Match identity (%)	References, acc. no.
51	+2	40778	41200	140	AAGGAAGTat**ATG**	-/ *Streptococcus thermophilus* phage Sfi21, hypothetical protein / 140	5e-85	90	Desiere et al., 1998, NP_049988
52	+3	41202	41624	140	AAAGATGTaatctaaa**ATG**	-/ *Streptococcus thermophilus* phage Sfi21, hypothetical protein / 140	1e-81	87	Desiere et al., 1998, NP_049989

[a] *da Silva Oliveira et al., 2004.*

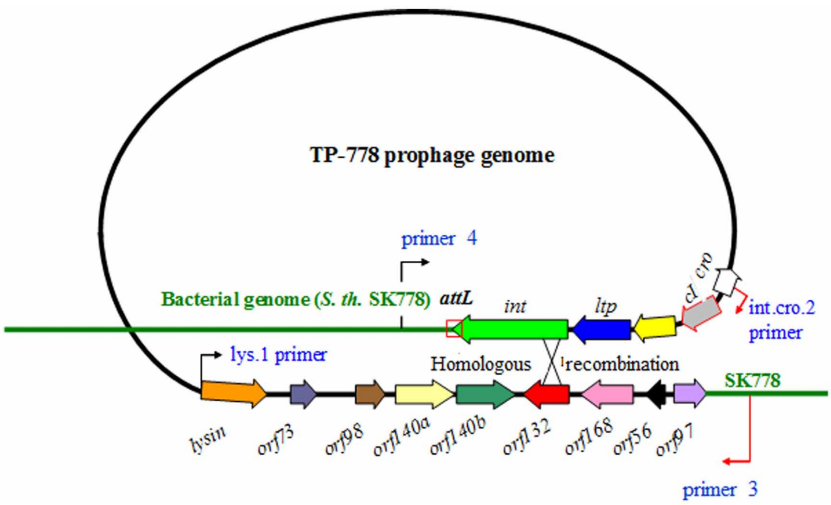

FIGURE 6 | Mechanism of excision of TP-778 prophage from its host's genome to yield phage TP-778L. Prophage and host DNA are shown by black and green line, respectively. Genes are indicated by arrows. Binding sites of primers 4 and 3 (Bruttin et al., 1997 are shown). The region of predicted cross-over is indicated by a cross.

the small (TerS) and the large terminase (TerL) units. The portal protein, encoded by the gene immediately following that of the large terminase, may be considered part of this module, however it also plays a critical role in head assembly (Padilla-Sanchez et al., 2013). The lack of similarity within the "DNA packaging" module only affects the N-terminal and central regions of TerS, which are involved in DNA binding and oligomerization, respectively (Sun et al., 2012). The C-terminal part, which is involved in interaction with the portal protein, is absolutely identical between TP-J34 and TP-778L. Thus, functionality defined as productive interaction with other components of the module is apparently not impaired by the alterations affecting TerS. The fact that both phages are *pac*-type phages and show high genome similarities to phages Sfi11 and O1205 confirms this finding. The last region of divergence between TP-J34 and TP-778L DNA concerns the "tail morphogenesis" module. Compared to the TP-J34 module, *orfs 45* and *48* appear to be fused to form the one large *orf42* of TP-778L. The gene product of *orf45* is characterized by a Lyz2 (Nambu et al., 1999) and a CHAP-domain (Bateman and Rawlings, 2003), indicating involvement in peptidoglycan hydrolysis during infection following adsorption. The gene product of *orf48* appears to be the receptor binding protein, containing a domain which is found in galactose-binding proteins (Gaskell et al., 1995). These three domains are found in the *orf42* gene product of TP-778L. It appears that both functions, which are required at the first steps of infection in TP-778, are combined in just one protein. This is not too surprising, since proteins encoded by genes with adjacent positions on the genetic map may also be in close contact within the structures formed. A fact that has been the basis for successful "block cloning" applied for elucidation of tail sub-structures (Campanacci et al., 2010).

The *orf48* gene product, containing the three 912 bp repeats, appears to be either physically unstable or inactive in the tail

assembly process. The few intact phage particles found after induction may arise from recombinational loss of the repeats occurring during replication: the few functional copies of Orf48 produced may initiate successful tail assembly. If TP-J34 DNA lacking the 912 bp repeat is packaged into such phage particles, TP-J34L phage particles are produced. The observed very low efficiency of plating for phage lysates resulting from induction of the prophage (Neve et al., 2003), even if they contained just one repeat may be due to phenotypic mixing (Streisinger, 1956), i.e., packaging of DNA into phage particles which are not derived from that DNA.

The 912 bp repeat shows DNA sequence homology to its flanking regions. However, an internal region of ca. 450 bp of the 912 bp repeat does not show homology to the flanking DNA or to other regions of TP-J34 DNA, which may indicate that this DNA region had been introduced by horizontal gene transfer. BlastN analysis revealed 80% sequence identity over the 450 bp to the host specificity gene of *S. thermophilus* bacteriophage DT2 (Duplessis and Moineau, 2001), and BlastX revealed 75% sequence similarity (*E*-value 2e-60) over 150 amino acids of the product of that gene. One may speculate that the DNA region has been obtained by horizontal gene transfer from a not yet identified phage with homology to phage DT2 in this genome region.

Horizontal gene transfer is apparently also responsible for the distribution of *ltp* genes, encoding a sie lipoprotein, among strains and bacteriophages of Gram-positive bacteria (Sun et al., 2006). The members of this family of "host cell surface-exposed lipoproteins" (Marchler-Bauer et al., 2011) are found scattered within annotated genomes of bacteriophage and bacteria (Sun et al., 2006). This would argue for *ltp* to be a member of the so called "morons," genes inserted into prophage genomes by horizontal gene transfer which provide some benefit to the host (Cumby et al., 2012). Further additional evidence for the "moron" character of *ltp* like presence of promoter and terminator will be presented elsewhere (Koberg et al., in preparation). The fact that the few temperate *S. thermophilus* phage harboring *ltp* are all very closely related indicates that horizontal transfer of an *ltp* gene into *S. thermophilus* phage occurred just once. The genome deviations seen among the three phages TP-J34, TP-778, and TP-EW should therefore have occurred after *ltp* had been acquired.

The differences in amino acid sequences and activities seen between plasmid-expressed $Ltp_{TP\text{-}J34}$ and $Ltp_{TP\text{-}778}$ confirm our recent data on $Ltp_{TP\text{-}J34}$ structure (Bebeacua et al., 2013), which indicated that the repeat domains are those responsible for super infection exclusion by interaction with the TMP of the super infecting phage and that the negatively charged amino acids in this region are important for interacting with the positively charged C-terminal end region of the P008 TMP. The deviations from $Ltp_{TP\text{-}J34}$ seen in the amino acid sequences of the $Ltp_{TP\text{-}778}$ repeat domain are mostly conservative. It is intriguing that with one exception the charges are not changed by the deviations. At this point it would just be speculation that the one change from negatively charged Glu to neutral Gly (see **Figure 8**) would be responsible for the functional differences. Another candidate for this difference could be the amino acid change from His to Pro (see **Figure 8**). However, this exchange does not affect a helix but just a ß-turn within the first repeat domain.

When discussing the potential effects on interaction with TMP of the amino acid exchanges seen between $Ltp_{TP\text{-}J34}$ and $Ltp_{TP\text{-}778}$, one should bear in mind that no genome sequence is available for lactococcal phage P001, a member of the c2-species. In the available genome sequence of lactococcal phage c2, however, no TMP is annotated (Lubbers et al., 1995). This is apparently due to the fact that phage c2 uses the host "phage infection protein" Pip for adsorption and DNA-injection (Monteville et al., 1994). In phage c2, gene 110 encoding the "tail adsorption protein" should be the TMP of phage c2. This protein would not

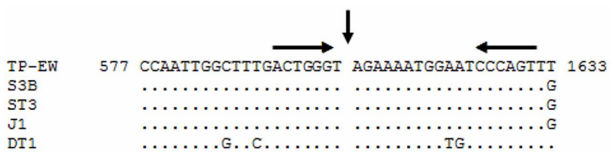

FIGURE 7 | Alignment of DNA sequences of *S. thermophilus* phages TP-EW, S3b (Acc. No. AF148561.1), ST3 (Acc. No. AF148565.1), J1 (Acc. No. AF148566.1), and DT1 (Acc. No. NC_002072) in the region surrounding the group-I-intron, present in all phage DNAs. The splice site is indicated by the vertical arrow. Sequence differences are indicated. Two 6-bp inverted repeats are indicated by horizontal arrows above the DNA sequence. The numbers flanking the TP-EW sequence correspond to the nt positions within the *lys* gene of this phage.

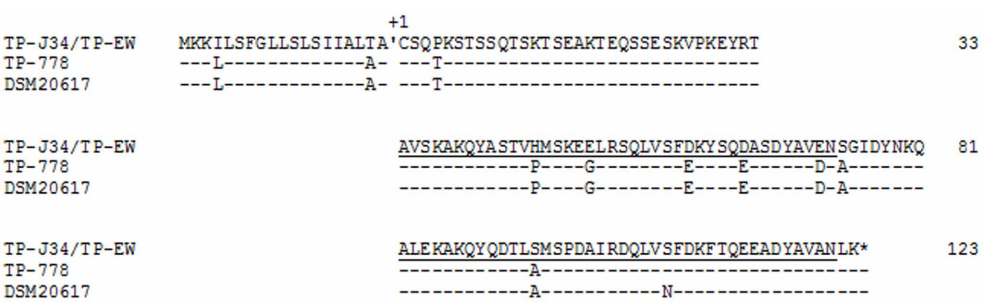

FIGURE 8 | Alignment of amino acid sequences of different Ltp proteins. The cleavage site between signal sequence and mature protein is indicated. The first Cys of the mature TP-J34 lipoprotein is marked as +1. The two repeat regions are underlined. Amino acids identical to those of TP-J34 are indicated by "-."

Table 5 | Plating efficiencies (E.o.p.) of lactococcal phages on *L. lactis* Bu2-60 expressing plasmid-encoded copies of $ltp_{TP\text{-}J34}$ or $ltp_{TP\text{-}778}$.

Plasmid	Gene expressed	E.o.p.		
		P008	P335	P001
pMG36e	–	1	1	1
pXMS2[a]	$ltp_{TP\text{-}J34}$	10^{-7} to 10^{-9}	0.7	0.7
pYAL1-3	$ltp_{TP\text{-}778}$	0.6	0.35	0.0001–0.1*

Means or ranges of at least three independently carried out assays are shown.
**Plaque sizes were significantly reduced.*
[a]Data from Bebeacua et al. (2013).

Table 6 | Semi-quantitative spottest for estimating the effects of different Ltp-proteins on infection of *L. lactis* Bu2-60 by different phage.

Phage	E.o.p. on *L. lactis* Bu2-60 expressing *ltp* gene			
	–	$ltp_{TP\text{-}778}$	$ltp_{TP\text{-}J34}$	$usltp1_{TP\text{-}J34}$
c2-SPECIES				
P001	1*	10^{-5}–10^{-6}	1	10^{-7}–10^{-8}, turbid
P197	1	10^{-6}–10^{-7}	1	10^{-6}–10^{-7}, turbid
P220	1	10^{-5}–10^{-6}	1	10^{-6}–10^{-7}, turbid
P624	1 (10^{9}–10^{10})	10^{-5}–10^{-6}, turbid	10^{-7}–10^{-8}	$<10^{-9}$
P653	1 (10^{9}–10^{10})	10^{-4}–10^{-5}, turbid	10^{-6}–10^{-7}, turbid	10^{-6}– 10^{-7}, turbid
P684	1 (10^{9}–10^{10})	1	10^{-5}–10^{-6}	10^{-5}–10^{-6}, turbid
936-SPECIES				
P008	1	1	10^{-7}–10^{-8}, turbid	$<10^{-9}$
P955	1	10^{-6}–10^{-7}	$<10^{-9}$	$<10^{-9}$
P957	1	1	10^{-2}–10^{-3}	$<10^{-9}$
P983	1	1	0.1–1	$<10^{-9}$
P993	1	10^{-6}–10^{-7}	$<10^{-9}$	$<10^{-9}$
P996	1	1	1	$<10^{-9}$
P335-SPECIES				
P335	1	1	1	$<10^{-9}$
P615	1	1	$<10^{-9}$	$<10^{-9}$

**If not indicated, titers of lysates were $>10^{10}$ pfu per ml. Deviating titers are shown in brackets.*

need to encompass the pore-forming function, since Pip provides this function. The fact that the secreted soluble $UsLtp_{TP\text{-}J34}$ is considerably less active against most phages attributed to the c2-species apparently underlines the peculiar situation of c2-phages with respect to TMP. With $UsLtp_{TP\text{-}J34}$ at hand, we may be able to test whether the "tail adsorption protein" is in fact the TMP of c2. At this stage, we can just notice that the C-terminal end of the c2 "tail adsorption protein" is positively charged, which is in agreement with the proposed binding site of $Ltp_{TP\text{-}J34}$ in TMP of P008 (Bebeacua et al., 2013).

To conclude, in this communication we could show that amino acid deviations seen between $Ltp_{TP\text{-}J34}$ and $Ltp_{TP\text{-}778}$ are apparently responsible for differences seen in the biological activities of both proteins. These deviations provide some clues on how to further study interaction between Ltp and TMP in more detail. Our data also show that phages TP-J34, TP-778, and TP-EW belong to the Sfi11 sub-species of *S. thermophilus* phages. The close relatedness of the three phages argues for acquisition of *ltp* prior to formation of the three phages from a common ancestor.

ACKNOWLEDGMENTS

We gratefully acknowledge technical assistance by I. Lammertz.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00098/abstract

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 December 2013; accepted: 23 February 2014; published online: 13 March 2014.

Citation: Ali Y, Koberg S, Heßner S, Sun X, Rabe B, Back A, Neve H and Heller KJ (2014) Temperate Streptococcus thermophilus phages expressing superinfection exclusion proteins of the Ltp type. Front. Microbiol. ***5****:98. doi: 10.3389/fmicb.2014.00098*

This article was submitted to Virology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 06 May 2014
doi: 10.3389/fmicb.2014.00201

Bacteriophage-insensitive mutants for high quality Crescenza manufacture

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Streptococcus thermophilus is a thermophilic lactic acid bacterium used as starter culture for the manufacture of fermented dairy products. For the production of Crescenza and other soft cheeses, Sacco has developed and provides dairies with three different defined blends of *S. thermophilus* strains. Each blend contains two different *S. thermophilus* strains. The strains were selected based on their unique technological properties as well as different phage profiles. Analysis of 133 whey samples collected in 2009–2010 from Italian dairies showed a high prevalence (about 50%) of bacteriophage attacks on the blend ST020. More specifically, the strain *S. thermophilus* ST1A was found to be the preferred target of the bacteriophages. A bacteriophage insensitive mutant (BIM5) of the phage-sensitive strain ST1A was successfully developed and used to substitute strain ST1A in the Crescenza starter culture ST020. The strain BIM5 showed identical technological and industrial traits as those of the phage-sensitive strain ST1A. The improved resistance of the modified Crescenza starter culture ST020R was confirmed at Italian dairies, and its effectiveness monitored on 122 whey samples collected in 2011–2012. Compared to the previous values (2009–2010), the use of the phage-hardened blend ST020R allowed reducing of frequency of phage attacks from about 50 to less than 5% of the whey samples investigated.

Keywords: ***Streptococcus thermophilus,*** **bacteriophages, bacteriophage-insensitive mutants, dairy fermentation, cheese, starter culture**

INTRODUCTION

Streptococcus thermophilus is the second most important industrial species after *Lactococcus lactis* (Fox and Cogan, 1993; Hols et al., 2005) and it is used in the manufacture of yogurt as well as many traditional Italian cheeses such as Mozzarella and Crescenza. In particular, Crescenza is a member of the "stracchino" cheeses family, but differs from the others since it is consumed fresh, without a ripening of about 20 days that is used for other members of the family. A schematic overview of the Crescenza manufacturing process is presented in **Figure 1**. Crescenza typical taste is milky, sweetish and mild, its paste is homogeneous and rather compact, pure white and buttery, melting in the mouth. The manufacture of Crescenza – and of cheese in general – requires the inoculation of milk with carefully selected bacterial strains blends, known as starter cultures, allowing the control of fermentation and production of high-quality fermented products. For the production of Crescenza, Sacco provides three blends of starter cultures each composed by two strains of *S. thermophilus.* Typically, the *S. thermophilus* strains are selected based on peculiar technological characteristics, such as low proteolytic ($PrtS^-$) and reduced mesophilic activities, as well as the ability to give the typical milky taste after fermentation.

Because significant amounts of bacterial cells are cultivated in the fermentation vats, most cheese industries have experienced problems with phage contaminations (Emond and Moineau, 2007). The first description of phages affecting a dairy starter culture was reported by Whitehead and Cox (1936), and even if since then the field has seen significant improvements, all over the world bacteriophages are still the major cause of dairy fermentations failures. Although raw milk is one of the source of phages in the industrial environment (Bruttin et al., 1997; Moineau and Lévesque, 2005), several dispersion pathways may occur in dairies. Personnel movements or transport of equipment and/or raw materials might cause the dispersion of phage particles as an aerosol (Verreault et al., 2011). In addition, phages present in recycled by-products may also spread to the entire factory environment, since they can remain in the air for long periods (Hinrichs, 2001). The first solution has been the alternate use (i.e., rotations) of starter cultures composed by strains that have different phage profiles (Thunell et al., 1985). In spite of this, phages are still causing significant losses in dairies and for this reason many studies are focusing on understanding phage-host dynamics, with special attention to bacterial resistance mechanisms (Allison and Klaenhammer, 1998; Labrie et al., 2010). The generation of Bacteriophage-Insensitive Mutants (BIMs), described by Mills et al. (2007), has received particular attention by starter culture manufacturers. It describes a relatively simple protocol that can be applied in any laboratory and that allows the generation of mutations in the sensitive microorganisms, that confer resistance to attacking phages. Increased resistance is typically achieved through mutations affecting bacterial-surface molecules interfering especially with phage attachment. BIMs are useful to fermentation-based industries; in fact it is possible to introduce them in the starter culture instead of the strains, which

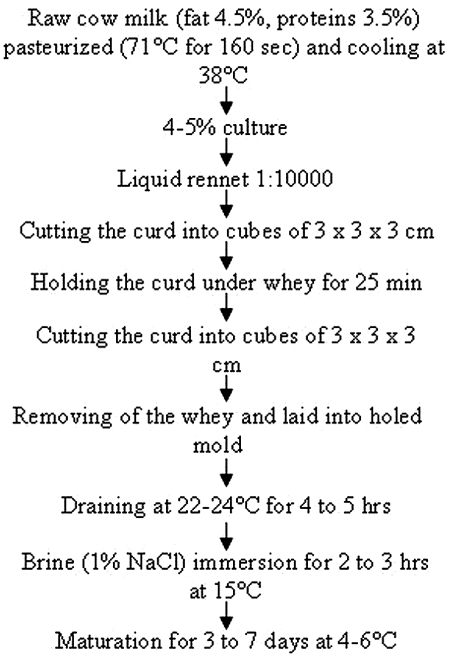

FIGURE 1 | Schematic representation of the Crescenza manufacturing process.

were sensible to a phage or a large number of phages, and this strategy increases the rotations robustness (Coffey and Ross, 2002).

Our phage analysis laboratory routinely investigate whey samples from dairy manufacturers, in order to detect the presence of phages, monitor performance of specific strains/starter cultures, as well as isolate new/novel bacteriophages. In particular, the whey samples of several Italian dairies producing Crescenza were controlled for 2 years, and the analysis of these results indicated that one starter culture, and particularly one *S. thermophilus* strain was showing high percentages of phage attacks. In this study, we have applied the BIM protocol described by Mills et al. (2007) to increase the phage resistance of the most sensitive *S. thermophilus* strains present in a Crescenza starter culture. The resulting mutant was used to generate a more robust starter culture, and its effectiveness was monitored over the following 2 years of use at Italian Crescenza manufacturers.

MATERIALS AND METHODS

CRESCENZA STARTER CULTURES, STRAINS AND CULTURE CONDITIONS

For Crescenza manufacture, Sacco produces 3 blends of PrtS$^-$ *S. thermophilus*, namely ST020, ST022 and ST024, each composed by 2 strains. In particular, the blend ST020 contains the strains ST1A and ST1B, the blend ST022 contains the strains ST2C and ST2D, and the blend ST024 contains the strains ST3E and ST3F. The BIMs generated from the strain ST1A were identified as BIM1, BIM2, BIM3, BIM4, BIM5, and BIM6.

Streptococcus thermophilus strains were routinely grown in 10% (w/v) skim milk powder (Irish dairy board, Dublin, Ireland) containing 0.1% (w/v) yeast extract (BD, Difco lab, Sparks, USA; LPEL broth) and heat-treated at 115°C for 15 min (LE). Frozen vials of each strain were inoculated at 2% (v/v) in 10 ml LPEL and incubated at 42°C until clotting. From this passage, the cell count (CFU/ml) was determined by serial dilutions on MRS agar (Oxoid, Hampshire, England) as described in the ISO method 27205 (2010).

pH-MULTISCAN ANALYSIS

Whey samples were collected from local dairies accounting for a total of 133 and 122, in the periods 2009–2010 and 2011–2012, respectively. Wheys were checked for phage content using a pH-Multiscan according to the manufacturer instructions (HNH Consult APS, Denmark). Briefly, 96-wells microtiter plates is inoculated with LE containing the two pH indicators bromocresol green and bromocresol purple. For each strain two wells are used, one containing 300 μl of LE and inoculated only with the strain under investigation (LES), and the second well containing 300 μl of LE inoculated with the strain as well as 0.3% of previously filtered (0.45 μm) whey (LEW). The analysis is performed in triplicate. The plates are incubated at 37°C, a scanner monitors the color (pH values) every minute, and a software translate the recorded values into acidification curves. A delay in the acidification curve of the LEW well compared to the LES well, indicates presence in the whey sample of phage/s attacking the inoculated strain.

BACTERIOPHAGES, PREPARATION AND ENUMERATION

The two *S. thermophilus* bacteriophages ϕ118 and ϕ215 were used to generate BIMs of the strain ST1A. The phages were considered different based on their host range, i.e., each of the phages attacks different *S. thermophilus* strains. Seventy-eight *S. thermophilus* phages of Sacco phage collection were used to check the phage profile of the mutants. These phages were isolated over the last 20 years from whey samples collected from different dairies mainly from Europe, South America and Asia.

Bacteriophage enumeration was achieved as follows. The strain ST1A was inoculated at 2% in 10 ml LE and 1% of the phages ϕ118 or ϕ215 were added. A control culture, without phages, was also prepared. Tubes were incubated at 42°C until clotting of the control. The samples with phages, which remained liquid, were added with 0.5% of lactic acid (sterilized at 121°C for 15 min), centrifuged at 4000 rpm for 15 min and then filtered through a 0.45 μm sterile filter. Bacteriophages were enumerated using the plaque test assay technique as described by Lillehaug (1997).

GENERATION OF BIMs AND DEVELOPMENT OF A NEW RESISTANT BLEND

Bacteriophage insensitive muntants of strain STA1 were generated and characterized by the 3-step process as described by Mills et al. (2007), using a multiplicity of infection of 10. The stability of the BIMs was controlled by growing each mutant in LE alone at 42°C for 10 passages (corresponding to circa 60 generations), and challenging the mutant with the phages ϕ118 and ϕ215. The colony morphology after the 10th passage was also compared to that of ST1A by streaking out on HHD agar as described by Camaschella et al. (1998). Acidification tests were performed in 9% (w/v) skim milk powder by continuous pH measurements with

Cinac4 (Alliance Instruments Dairy Division, France) as described in the ISO method 26323 (2009). Acidification data of the BIMs were compared with those of the strain ST1A. The resistance of the BIMs against 80 *S. thermophilus* phages was investigated using the pH-Multiscan as described above. The generated BIMs were also compared to the parental strain, by using Pulsed Field Gel Electrophoresis (PFGE), using the restriction enzyme SmaI as described by Mills et al. (2007). The BIMs more similar to the strain ST1A were selected for pilot scale production, blended at 50% ratio with the strain ST1B, and the acidification performance of the resulting starter culture ST020R was compared to that of ST020 using the Cinac4 as described above.

TECHNOLOGICAL TEST OF THE NEW BLEND

To verify if the blend ST020R containing the BIM of ST1A was suitable for the production of high quality Crescenza, at a pilot plant of a local dairy two Crescenza productions were performed, one with ST020 and one with ST020R. Briefly, 2000 L of cow milk (4.5% fat and 3.5% proteins) were heat-treated at 71°C for 160 s and then cooled to 38°C. The starter culture and liquid rennet 1:10000 (Caglificio Clerici, Cadorago, Italy) were added and incubated at 38°C for 30 min. The curd was broken in two phases into cubes of 3 cm × 3 cm × 3 cm, with a rest of ca 25 min in between. The mass was mixed again, the whey was removed, laid in holed mold, i.e., square plastic boxes with sides of 20 cm, and stewed for 4–5 h at 22–24°C. The curd was performed by immersing the cheese in brine (1% NaCl) for 2–3 h at 14–16°C. The cheese was matured for 3–7 days. The Crescenza produced with either starter culture was tasted by a professional team of sensorial evaluators.

RESULTS

ANALYSIS OF WHEYS AND IDENTIFICATION OF THE STRAIN TO BE HARDENED

Routinely, whey samples from dairy customers are analyzed with the method described in section "pH-Multiscan Analysis." All the data collected are used to monitor efficacy of the starter cultures used, which is expressed as frequency of whey samples containing phages able to attack the strains present in the starter cultures. Concerning the blends ST020, ST022, and ST024 sold for Crescenza manufacture, the results of attacks on single strains contained in each blend are summarized in **Figure 2A**. In particular, the analysis of 68 and 65 samples collected in 2009 and 2010, respectively, showed high frequencies of attacks for the blend ST020, and more specifically for the strain ST1A. The second most attacked strain was ST2C, used in ST022. Strain ST1A was therefore selected to be hardened against the two phages ϕ118 and ϕ218 isolated from whey samples, and previously inserted in Sacco phage collection.

GENERATION AND CHARACTERIZATION OF BIMs

Eight BIMs (BIM1 to BIM8) of ST1A were generated using the 3-step process described by Mills et al. (2007). The BIMs and ST1A were subjected to PFGE analysis, stability and acidification tests as well as phage profile by using the 80 *S. thermophilus* phages available. The results of the performed test are summarized in **Table 1**. PFGE analysis of the DNA isolated from the 8 BIMs revealed an almost identical profile to that of ST1A (**Figure 3**), apart from the lack of one band (white arrow, **Figure 3**). To determine stability of the acquired phage-resistance, the BIMs were grown for about 60 generations without selective pressure, i.e., presence of phages. When exposed to the presence of the phages ϕ118 and ϕ218, only six out of the eight generated BIMs maintained their resistant profile, and were thus subjected to the acidification test (**Table 1**). Comparing the acidification performance of the six stable BIMs to that of the phage-sensitive strain ST1A revealed that only BIM1, BIM4, BIM5, and BIM6 maintained an acidification activity similar to that of ST1A (data not shown), and they were thus selected for further analysis. The four selected BIMs were controlled against further 80 phages, and only two of them, namely BIM4 and BIM5, were found resistant to all investigated phages (**Table 1**). These two BIMs were produced at the pilot plant, and used for further tests.

LABORATORY FORMULATION OF A CRESCENZA STARTER CULTURE CONTAINING A BIM

When produced at the pilot plant, BIM4 and BIM5 showed comparable growth rate and overall yield to that of the strain ST1A (data not shown). Both mutants were produced as frozen pellets, and their acidification performance was investigated as described above. The data collected revealed an acidification profile similar to that of the parental strain ST1A (**Figure 4A**). For simplicity, the strain BIM5 was selected and used to substitute the strain ST1A in the blend ST020, giving rise to the new blend ST020R.

Using the acidification protocol described in paragraph 2.5, the performance of the phage-hardened blend ST020R was found identical to that of the phage-sensitive blend ST020 (**Figure 4B**).

CRESCENZA MANUFACTURE WITH THE PHAGE-HARDENED STARTER CULTURE ST020R

Having proven that the performance of the phage-hardened starter culture ST020R was identical to that of ST020 under laboratory conditions, the next step was to prove its suitability for production of high quality Crescenza at a local Crescenza manufacturer. To reduce the variability due to different lots of cheese milk, on the same day 2000 L of cheese milk was fermented either with ST020 (control) or with the phage-hardened blend ST020R. During production no significant difference was observed when using the blend ST020R (data not shown), and also a sensory evaluation throughout the shelf life of the Crescenza produced did not reveal significant differences between the Crescenza produced with ST020 or ST020R starter cultures. The new blend ST020R was used at the same inoculation level as the blend ST020, and it did not contain a higher *S. thermophilus* cell count (data not shown).

CONTINUOUS MONITORING OF THE PHAGE-HARDENED BLEND EFFICACY

The improved blend ST020R substituted the phage-sensitive blend ST020. Routine monitoring of the whey samples arriving from different dairies continued over the years 2011 and 2012. The percentage of whey samples analyzed and found to contain phages able to attack the strains present in the 3 Crescenza starter cultures

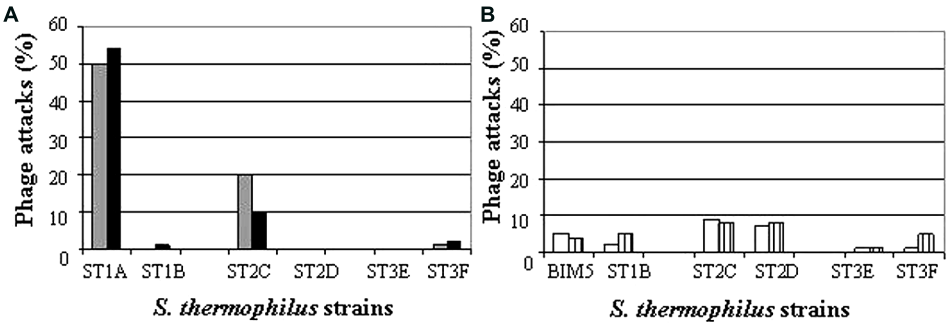

FIGURE 2 | Frequency of phage attacks of the strains contained in the standard Crescenza blends ST020 (ST1A and ST1B), ST022 (ST2C and ST2D), and ST024 (ST3E and ST3F) as well as the phage-hardened blend ST020R (BIM5 and ST1B). Each strain was grown in milk or in milk with whey sample. A delay in growth rate of a strain by the addition of whey was considered as a phage attack. **(A)**, whey samples collected during the years 2009 (grey) and 2010 (black); **(B)**, whey samples collected during the years 2011 (white) and 2012 (vertical line pattern).

Table 1 | Genetic and physiological tests performed on the eight BIMs generated from the phage-sensitive strain ST1A.

	Analysis type			
BIM code	**PFGE profile**	**Stability[a]**	**Acidification**	**Phage profile against 78 phages[b]**
BIM1	Similar to ST1A	Stable	Similar to ST1A	Sensitive[c]
BIM2	Similar to ST1A	Stable	Slow	n.d.
BIM3	Similar to ST1A	Stable	Slow	n.d.
BIM4	Similar to ST1A	Stable	Similar to ST1A	Resistant[d]
BIM5	Similar to ST1A	Stable	Similar to ST1A	Resistant
BIM6	Similar to ST1A	Stable	Similar to ST1A	Sensitive
BIM7	Similar to ST1A	Instable	n.d.	n.d.
BIM8	Similar to ST1A	Instable	n.d.	n.d.

[a] *Assessed by growing the BIMs without phages for ca. 60 generations and thereafter exposing cells to the phages ϕ118 and ϕ218.*
[b] *All phages from the phage collection were used to assess the sensitivity of the BIMs.*
[c] *Attacked by at least 1 phage out of the 78 tested.*
[d] *Resistant to all 78 phages.*

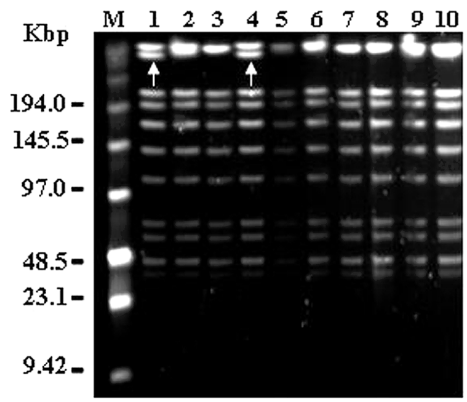

FIGURE 3 | Pulsed Field Gel Electrophoresis profiles of the phage-sensitive strain ST1A and its generated BIMs. The enzyme used for digestion is SmaI. ST1A, lane 1 and 4; BIM1, lane 2; BIM2, lane 3; BIM3, lane 5; BIM4, lane 6; BIM5, lane 7; BIM6, lane 8; BIM7, lane 9; BIM8, lane 10. The white arrow indicates the band that is missing in the BIMs profiles.

was measured as shown in **Figure 2B**. The analysis of 64 and 58 whey samples collected in 2011 and 2012, respectively, showed a low percentage of attacks in particular for the strains present in ST020R. In particular, the BIM5 clearly revealed a lower percentage of phage attacks when compared to the values recorded for ST1A, allowing to reduce the frequency of phage attacks from ca. 50% for the blend ST020 in the years 2009–2010 to less than 5% for the blend ST020R in the years 2011–2012. The new blend ST020R was used at the same inoculation level of the blend ST020, and it did not contain a higher *S. thermophilus* cell count (data not shown).

DISCUSSIONS

Virulent lactic acid bacteria phages are the cause of serious problems during manufactures of dairy products, and industries are constantly waging war against these viruses to keep them under control. In the Italian productions of cheese and yogurt, phage infections of *S. thermophilus* are becoming a more persistent problem. While supplying 3 starter cultures to Crescenza manufacturers, we observed over a 2 years period that the strain ST1A contained in the blend ST020 was frequently attacked by phages (**Figure 2A**). Our results were confirmed by the cheese manufacturers, who experienced several production problems when using this particular blend in the period under investigation (data not shown). By using the BIM protocol described by Mills et al. (2007) we phage-hardened the strain ST1A, and selected the BIM5 as a substitute of the parental strain in the blend ST020. The use of the resulting phage-hardened blend ST020R allowed to significantly reduce the frequency of phage attacks (circa 10-fold) when monitoring whey samples collected over the following 2 years (**Figure 2B**).

The identification of a phage sensitive strain within starter cultures is a common experience for starter culture manufacturers, and it is often linked to the frequency of use of the strain/blend

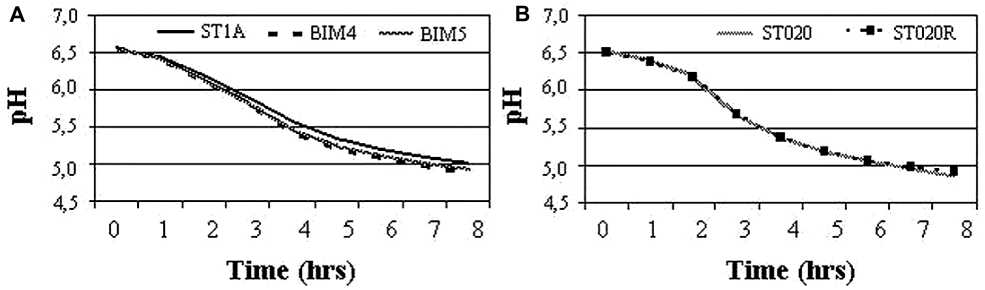

FIGURE 4 | Acidification profiles of the phage-resistant strains BIM4 and BIM5 produced at the pilot plant as frozen pellets, compared to that of frozen pellets of the phage-sensitive strain ST1A (A). Acidification performance of the blend ST020R containing the BIM5, compared to that of the phage-sensitive blend ST020 **(B)**.

at a particular dairy. Phage collections are commonly used to characterize and differentiate strains that are included in culture collections, and thus one simple solution would seem the replacement of a phage-sensitive strain with a phage-insensitive from the culture collection. However, many important technological traits are strain-specific and therefore the replacement is often not possible or desirable (Capra et al., 2011). Furthermore, the industrialization of a novel strain requires high investments, due to scaling up and optimization of fermentation conditions, and there is no assurance that the strain selected as replacement will be resistant to phages present in the dairies. To overcome this problem, Mills et al. (2007) published an efficient and low-cost three step method of isolating BIMs of *S. thermophilus* by multiple passages in milk in the presence of phages against which protection is required. There are many other published methods for spontaneous BIM generation. Generally, they involve the challenge of cultures with high levels of single (Coffey et al., 1998; Guglielmotti et al., 2009) or multiple (Marcó et al., 2011a,b) bacteriophages, and they have different degrees of success depending on the species and strain considered. BIMs of the original strain may be isolated which are resistant to the offending phage while retaining their technologically important attributes. For this reason, when applying Mills et al. (2007) protocol we have included several control steps of the generated BIMs (**Table 1**), to assure that no major genetic reassembly occurred (**Figure 3**), that the growth in milk and that the performance of the starter culture was still maintained both under laboratory conditions (**Figure 4B**) as well during Crescenza manufacture. Of the eight BIMs obtained, only two maintained the desirable traits of the strain ST1A. The loss of stability of the phage-resistant phenotype that we observed for BIM7 and BIM8 has already been observed (Mahony et al., 2012). Changes in cell wall polysaccharides structure or mutations in key biosynthetic genes are expected to cause detrimental effects on growth and/or cell division, which might explain the reduced growth phenotype we observed for BIM2 and BIM3 when they were inoculated in laboratory milk (data not shown). Remarkably, BIM1 and BIM6 showed to have become sensitive against other phages present in the phage collection, thus underlining the importance of controlling that all desirable traits of the phage-sensitive strain are maintained in the generated BIMs.

Unfortunately, bacteriophages rapidly evolve (due to high frequency of mutations) to overcome any changes in the host receptor and/or resistance mechanisms of their target strains. Analysis of phage mutants with expanded/altered host ranges demonstrates that point mutations in structural tail genes is the sole requirement to overcome changes in host receptors, improve host adsorption or to infect new strains (Ravin et al. , 2002; Duplessis et al., 2006; Uchiyama et al., 2011). As such mutations can rapidly occur, there is no indication and assurance concerning the duration of the robustness of a particular BIM/starter culture in the dairies. In the industrial praxis, some BIMs have shown to confer resistance for several years, as we have clearly described in this work, whereas other failed within few months of applications (own observations, data not shown). Understanding the evolutionary and molecular processes that allow phages to overcome host barriers may provide novel indications for the development of the next generation of phage-resistant starter cultures. For the time being, starter culture manufacturers will be daily challenged to produce and provide high quality starter cultures that can compete and resist a quickly evolving bacteriophage community. Accurate strain selection and starter culture development, correct use of starter culture rotations as well as strict hygiene conditions in the dairies will still remain the key steps for the successful production of high quality dairy products such as Crescenza.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 15 November 2013; accepted: 16 April 2014; published online: 06 May 2014. Citation: Chirico D, Gorla A, Verga V, Pedersen PD, Polgatti E, Cava A and Dal Bello F (2014) Bacteriophage-insensitive mutants for high quality Crescenza manufacture. Front. Microbiol. **5**:201. doi: 10.3389/fmicb.2014.00201*

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 22 May 2014
doi: 10.3389/fmicb.2014.00236

Interactions of the cell-wall glycopolymers of lactic acid bacteria with their bacteriophages

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Lactic acid bacteria (LAB) are Gram positive bacteria widely used in the production of fermented food in particular cheese and yoghurts. Bacteriophage infections during fermentation processes have been for many years a major industrial concern and have stimulated numerous research efforts. Better understanding of the molecular mechanisms of bacteriophage interactions with their host bacteria is required for the development of efficient strategies to fight against infections. The bacterial cell wall plays key roles in these interactions. First, bacteriophages must adsorb at the bacterial surface through specific interactions with receptors that are cell wall components. At next step, phages must overcome the barrier constituted by cell wall peptidoglycan (PG) to inject DNA inside bacterial cell. Also at the end of the infection cycle, phages synthesize endolysins able to hydrolyze PG and lyse bacterial cells to release phage progeny. In the last decade, concomitant development of genomics and structural analysis of cell wall components allowed considerable advances in the knowledge of their structure and function in several model LAB. Here, we describe the present knowledge on the structure of the cell wall glycopolymers of the best characterized LAB emphasizing their structural variations and we present the available data regarding their role in bacteria-phage specific interactions at the different steps of the infection cycle.

Keywords: lactic acid bacteria, bacteriophage, cell wall, phage receptor, endolysin, polysaccharide, peptidoglycan, teichoic acid

INTRODUCTION

The cell wall of Gram-positive bacteria which surrounds the cytoplasmic membrane is a complex arrangement of different biopolymers: peptidoglycan (PG), polysaccharides, teichoic acids and (glyco)proteins (Delcour et al., 1999) (**Figure 1A**). PG is the major component of the Gram-positive cell wall and it is made of glycan chains cross-linked through short peptide chains. It constitutes a network around the bacterial cell on which are linked covalently secondary polymers such as wall teichoic acids (WTA), polysaccharides, or LPXTG-containing proteins. Proteins can also be attached non-covalently by recognizing specific motifs of cell wall polymers or they can be organized as a layer outside the cell (S-layer). Lipoteichoic acids (LTA) anchored in the cytoplamic membrane and inserted in the cell wall contribute also to its properties and functions. The major role of the cell wall is to maintain bacterial shape and integrity. In addition, its components exposed at the bacterial surface constitute the first line of molecules to interact with abiotic or biotic environment, including eukaryotic host cells and bacteriophages.

Lactic acid bacteria (LAB) are Gram-positive bacteria widely used in food fermentations due to their ability to convert sugars into lactic acid. Lactococci and lactobacilli are used as starters in milk fermentations for the production of cheese and yogurts. They acidify milk through lactic acid production which limits food spoilage and in addition they contribute to the development of organoleptic properties including texture and flavor (Lortal and Chapot-Chartier, 2005). Bacteriophages infecting LAB constitute a real threat for dairy fermentations. Lysis of starter bacteria during their growth leads to slow or failed milk acidification, to poor quality products and finally to economic losses (Garneau and Moineau, 2011). It is expected that a better understanding of the molecular mechanisms of bacteriophage interactions with their host strain will provide new strategies to control phage infections.

During the phage infection cycle, the bacterial cell-wall components which possibly show considerable variations between species and strains are key determinants of the specific interactions of bacteriophages with their target bacteria (Samson and Moineau, 2013) (**Figure 1A**). First, bacteriophage particles must attach to bacteria and at this early step, cell-surface-exposed components of the bacterial wall are the likely recognized receptors (Forde and Fitzgerald, 1999). Then, phages must inject their DNA inside the bacterial cell and this step may be facilitated by PG-hydrolases (PGHs), able to locally degrade PG to make small-size holes inside the wall and allow safe passage of DNA injection device to the cytoplasmic membrane without lysing bacterial cells (Kenny et al., 2004). Finally, at the end of the infection cycle, bacteriophages make the infected cells burst to release the phage progeny; this step generally occurs by synthesis of phage-encoded PGHs, named endolysins, which recognize specifically and hydrolyze the bacterial cell wall PG (Oliveira et al., 2013).

A growing interest for the structure and function of the cell-wall glycopolymers of LAB has emerged in the past years due to their potential involvement in LAB functionality including

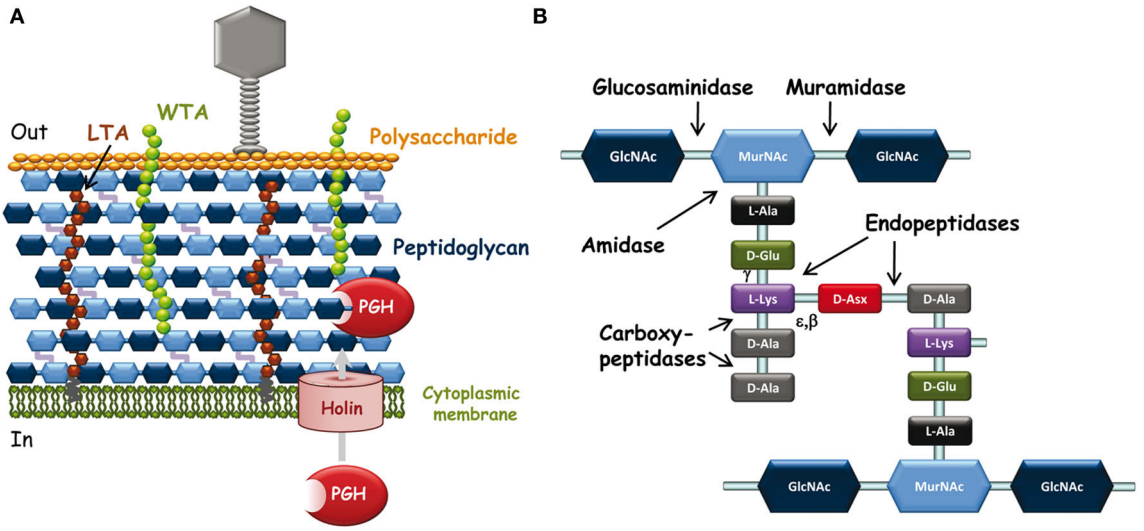

FIGURE 1 | (A) Schematic representation of *L. lactis* cell wall and its interactions with infecting bacteriophages. Outside the bacterial cell, phages adsorb to bacteria through specific recognition of receptors (polysaccharide on this scheme) located at the bacterial surface. Inside the bacterial cell, at the end of the infection cycle, endolysin and holin encoded by the phage genome are synthesized to lyse bacteria and release phage progeny. Endolysins are PG-hydrolases that gain access to their substrate by passing through pores made by oligomerization of holins. **(B)** Schematic peptidoglycan structure. Structure with D-Asx cross-bridge (A4α-chemotype) is found in *L. lactis* and in several *Lactobacillus* species. Asx stands for Asp or Asn. The third amino acid of the stem peptide (L-Lys) may be replaced by mDAP (in *L. plantarum*) and the fifth D-Ala of the peptide stem by D-Lac in certain lactobacilli such as *L. casei*. The cross-bridge is also variable between bacterial species. The cleavage sites of the different types of PG-hydrolases are indicated by arrows. Muramidase, N-acetyl-muramidase; glucosaminidase, N-acetyl-glucosaminidase; amidase, N-acetyl-muramyl-L-Ala-amidase; endopeptidases with γ-D-Glu-L-Lys-endopeptidase or D-Ala-D-Asp-endopeptidase (specific of cross-bridge) specificity. Carboxypeptidases including D,D-carboxypeptidases and L,D-carboxypeptidases are involved in PG maturation; they were not found among endolysins.

bacterial growth and fitness, interactions with their eukaryotic host in the case of commensal and probiotic strains and sensitivity to bacteriophages. In this review, we summarize the current knowledge on the different cell wall glycopolymers including polysaccharides, teichoic acids and PG, studied mainly in four model LAB species: *Lactococcus lactis*, *Lactobacillus plantarum*, *Lactobacillus casei*, and *Lactobacillus rhamnosus.* For each component type, we present the available data regarding their role in bacteriophage infection cycle.

CELL-WALL GLYCOPOLYMERS AS RECEPTORS OF BACTERIOPHAGES

The first step of bacteriophage infection is the adsorption of the phage particles to the bacterial host. This event involves recognition by phage receptor-binding proteins (RBPs) of receptors located on the target bacterial cell surface. Regarding LAB phages, until now previous studies have identified proteins as well as non-proteinaceous compounds of the cell wall such as polysaccharides or teichoic acids as phage receptors (Mahony and van Sinderen, 2012). The receptor for the C2-type group of phages infecting *L. lactis* was previously identified to be the membrane protein termed Pip (phage infection protein); adsorption of c2-phage follows a two-step process with reversible saccharide binding prior to irreversible binding to Pip protein (Geller et al., 1993; Monteville et al., 1994). In the following text, we will focus on non-proteinaceous cell wall glycopolymers identified as phage receptors.

CELL-WALL POLYSACCHARIDES IN LAB

The polysaccharidic components of Gram-positive bacteria surface may be divided into three groups: (i) capsular polysaccharides (CPS) that are, in most cases, covalently bound to PG and form a thick outer layer named capsule; (ii) wall polysaccharides (WPS) that may be attached to the cell wall whether or not covalently, but without forming a thick capsule; and (iii) extracellular polysaccharides (EPS) which are released into the cell environment and are not attached to the cell surface. Different polysaccharides may be produced by the same bacterium (Caliot et al., 2012), although at the experimental level it may be difficult to differentiate unambiguously the different groups.

A WPS, which is not an EPS and capable of forming an outer layer at the bacterial surface, was discovered in *L. lactis* MG1363 (Chapot-Chartier et al., 2010). The WPS chains are composed of hexasaccharide-phosphate repeating units (**Figure 2**), which are distinct from other bacterial polysaccharides. Also it differs from previously characterized *L. lactis* EPS and is most probably covalently attached to the cell wall as regard the harsh acid treatment used to detach it from the bacterial cells. Atomic force microscopy (AFM) allows exploring bacterial surface architecture at the nanoscale level and was recently used to probe the surface of several LAB, including *L. lactis*, *L. plantarum*, and *L. rhamnosus* (Tripathi et al., 2012). In *L. lactis* MG1363, AFM as well as complementary transmission electron microscopy (TEM) observations show that the characterized WPS forms a compact outer layer surrounding the cell which was named pellicle

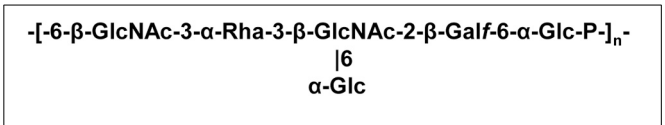

FIGURE 2 | Structure of sugar-phosphate polysaccharide pellicle of *L. lactis* MG1363.

(Chapot-Chartier et al., 2010). It was visualized as an electron dense layer by TEM and as a smooth layer by AFM around the cells. A derivative mutant lacking this WPS layer was obtained and was found to have a rough surface by AFM. In addition, by imaging the surface of this WPS-negative mutant with a tip functionalized with the PG-binding LysM domain, PG could be imaged as parallel cables around the bacterial cells (Andre et al., 2010). It is worth noting that a similar outer layer can be observed in a number of TEM micrographs of *L. lactis* strains of different origins, although its existence was not reported (Chapot-Chartier et al., 1994; Dabour et al., 2006). In *L. lactis* MG1363, a WPS-negative mutant makes long chains of cells which appear to have morphological defects. These observations suggest that WPS is required for normal cell division and separation. Also the WPS layer was shown to protect bacteria from phagocytosis by macrophages (Chapot-Chartier et al., 2010).

The synthesis of this WPS is encoded by a large cluster of genes in MG1363, which is conserved among *L. lactis* strains but exhibits genetic diversity that was recently analyzed in details (Mahony et al., 2013a).

Other polysaccharides associated to the cell surface were described in lactobacilli. In *L. plantarum* WSF1, four gene clusters associated with polysaccharide production are encoded in the genome (Remus et al., 2012). All these four gene clusters contribute to the overall surface polysaccharides produced by *L. plantarum*. However, in this case, the structure of the different polysaccharides has not been established until now. The surface polysaccharides were shown to influence the immunomodulatory properties of the wild-type strain probably by reducing the release or the exposure of activating molecules of the bacterial surface.

In *L. rhamnosus* GG, a long galactose-rich polysaccharide was found at the bacterial surface (Lebeer et al., 2009). This polysaccharide named EPS was detected at the bacterial surface of LGG by AFM and contributes to bacterial cell surface properties which determine adhesion and biofilm formation (Francius et al., 2009). The structure of this polysaccharide most probably corresponds to the one described earlier (Landersjo et al., 2002). The gene cluster specifying this polysaccharide in LGG exhibits differences with the clusters identified in other strains of *L. rhamnosus* in agreement with different composition of the synthesized polysaccharides (Peant et al., 2005). When the cell surface of *L. rhamnosus* was explored by AFM, it revealed a rough morphology decorated with waves (Francius et al., 2009). In contrast, a WPS-negative mutant showed a much smoother morphology suggesting that these wave-like structures reflect the production of WPS. In addition single molecule force spectroscopy with lectin-modified tips, revealed the existence of polysaccharide chains of different nature at the cell surface, polysaccharide rich in mannose or glucose having moderate extension and polysaccharide rich in galactose with much longer extensions. Deprivation of bacteria of the long galactose-rich polysaccharide results in an increased adherence and ability to form biofilm suggesting that surface adhesins such as pili structures were demasked at the bacterial surface (Lebeer et al., 2009). In addition, this polysaccharide has a protective role against host immune antimicrobial peptides (Lebeer et al., 2011).

In *L. casei* Shirota strain, two types of WPS were also described: longer, high molecular mass PS-1 and shorter low molecular mass PS-2. The gene cluster encoding PS-1 biosynthesis was identified (Yasuda et al., 2008) and PS-1 structure was previously determined (Nagaoka et al., 1990). The glycome of *L. casei* strains was compared with a lectin microarray and allowed to evidence different profiles between strains suggesting different WPS (Yasuda et al., 2011). In *L. casei* Shirota, WPS was shown also to have an immune suppressive function (Yasuda et al., 2008).

Finally, the diversity of WPS between strains of the same species was also recently observed in *Lactobacillus helveticus* strains and it was hypothesized that these different polysaccharide structures could contribute explaining the different autolytic properties observed between the studied strains (Vinogradov et al., 2013).

As a conclusion, WPS appear as omnipresent components of the cell surface of LAB and exhibit most probably high structural diversity between strains the same species.

CELL-WALL POLYSACCHARIDES AS BACTERIOPHAGE RECEPTORS IN LACTOCOCCI

L. lactis phages are the best characterized and numerous individuals were isolated because of the wide use of *L. lactis* in dairy industrial fermentations (Garneau and Moineau, 2011). They were previously classified in 10 groups on the basis of their lytic activity on a range of *L. lactis* strains, morphology or more recently DNA-DNA hybridization and multiplex PCR. The predominant *L. lactis* phages are found in three main groups: 936, c2, and P335 species which belong to the Siphoviridae phage family, the most problematic infecting *L. lactis* and certain *Lactobacillus* species. The 936 phages are strictly lytic and thus received more specific attention because they are threatening dairy fermentations involving *L. lactis* starters (Mahony et al., 2013b). However, inside the wide 936 group, phages differ at the level of their RBPs and thereby potentially at the level of their host range (Mahony and van Sinderen, 2012).

Initial studies conducted to identify the phage receptor of 936-phages indicated that a bacterial cell-wall component differing from a protein and containing rhamnose was involved in adsorption of the phage at the bacterial surface (Valyasevi et al., 1990). Further studies using transposon random mutagenesis allowed to identify genes required for adsorption of two 936-type bacteriophages to their respective host strain. Mutations were mapped inside a gene cluster potentially involved in WPS biosynthesis (Dupont et al., 2004). Later on, the WPS of *L. lactis* MG1363 named pellicle was discovered, its structure determined and it was shown to be encoded by the corresponding gene cluster in MG1363 genome (Chapot-Chartier et al., 2010). In addition, a pellicle-negative mutant was shown to be resistant to the 936-bacteriophage sk1 strongly suggesting that this WPS consisting in hexasaccharide subunits bound through phosphodiester bonds, could be the sk1 phage receptor.

In a recent study, the gene cluster encoding WPS biosynthesis in various *L. lactis* strains was shown to contain both highly conserved regions as well as regions of high diversity, suggesting that WPS structure could be a variable character between strains (Mahony et al., 2013a). Detailed analysis of the proteins encoded in the gene cluster allowed the classification of *L. lactis* strains in three subgroups (CWPS type A, B, and C) based on the diversity regions. In parallel, a panel of 936-type phages infecting *L. lactis* was classified in different groups according to their host range and their encoded RBP sequence. Mahony et al. (2013a) revealed a correlation between the pellicle genotype of a given host strain and the host range of the tested 936-type phages. These results support the proposed role of WPS pellicle as 936-phage receptor and variations of its structure could explain the narrow host range of this type of phages. This hypothesis was very recently confirmed by the structure determination of the WPS purified from a second *L. lactis* strain with a different WPS-pellicle genotype. WPS from *L. lactis* strain 3107 was shown to be composed of pentasaccharide repeating units linked by phosphodiester bonds and thus differs from the WPS characterized in *L. lactis* MG1363. In addition, this WPS was shown to be the receptor used by several 936- phages infecting *L. lactis* 3107 (Ainsworth et al., 2014).

Remarkably, in parallel studies, the 3D-structure of the receptor binding proteins (RBPs) (also sometimes named anti-receptors) has been elucidated in several cases, including those of 936-phages p2 and bIL170 as well as P335-like phage TP901-1 (Ricagno et al., 2006; Spinelli et al., 2006a,b). These RBPs are localized at the tip of the phage tail and allow the phage to recognize specifically its receptor at the bacterial surface. The crystal structure of the protein complex connecting the RBP to the rest of the phage tail was also solved for siderophages p2 and TP901-1 (Sciara et al., 2010; Veesler et al., 2012). Recently the binding of RBP to the WPS pellicle was demonstrated in the case of the p2 RBP with the purified pellicle from its host strain MG1363 with the use of surface plasmon resonance (SPR) (Bebeacua et al., 2013). The RBP from the P335-phage TP901 which does not infect MG1363 exhibited a much lower affinity for the MG1363 pellicle. The specificity was shown to result mainly from a lower k_{off} value of the RBP/saccharide dissociation.

TEICHOIC ACIDS IN LAB

Teichoic acids are phosphate-rich glycopolymers that are classified into two groups: LTA anchored in the cytoplasmic membrane through a glycolipid and WTA covalently bound to PG. In certain Gram-positive bacteria such as *Bacillus subtilis*, WTA may represent up to 50% of the cell wall dry mass (D'Elia et al., 2006). WTA are quite diverse in structure but the most common ones are polymers of glycerol-phosphate (poly(Gro-P)) or ribitol-phosphate (poly(Rbo-P)) (**Figure 3**). With respect to LTA, the most common structure is also a poly(Gro-P) chain. It is worth noting that LTA and WTA have different biosynthetic pathways, even if they are made of similar repeating units such as Gro-P (Weidenmaier and Peschel, 2008). The glycerol or ribitol chains may be substituted with D-alanyl- or glycosyl-residues (e.g., Glc, Gal, GlcNAc) which contribute to teichoic acid functionality. In particular, D-alanyl residues provide their positive charges as counter ions of negative phosphate groups and modify the physico-chemical environment inside the cell wall and/or at the bacterial surface (Neuhaus and Baddiley, 2003).

Due to their polyanionic nature and their abundance, both WTA and LTA play multiple and varied roles in bacterial physiology. They are involved in regulation of ion homeostasis inside the cell wall, in modulating autolytic activity and in controlling cell division and morphogenesis. Also they are crucial for bacteria host interactions since their D-alanylation protect bacteria against cationic antimicrobial peptides. They also influences bacterial adhesion to abiotic surfaces and to host cells. Finally, they are recognized by the host as molecular-associated microbial patterns (MAMPS) (Brown et al., 2013; Schneewind and Missiakas, 2014).

In *L. casei or L. rhamnosus*, no WTA were detected in agreement with the absence of *tag* or *tar* biosynthesis genes, whereas in *L. lactis*, the presence of WTA remains to be further investigated. WTA have been described in *L. plantarum* strains which appear to produce either poly(Gro-P) or poly(Rbo-P) WTA. Moreover, several *L. plantarum* strains contain the genes to synthesize the two types of WTA (Bron et al., 2012).The cell surface of *L. plantarum* was also investigated by AFM combined with fluorescence microscopy with specific lectin probes (Andre et al., 2011). This approach combined with the use of specific cell-wall mutants devoid of WPS or WTA, allowed imaging the distribution of WTA at the bacterial surface. In this way it was shown that wild-type cells have a highly polarized surface morphology with smooth poles and rough lateral regions. Together with fluorescence labeling with lectin probes, AFM showed that WTA are heterogeneously distributed at the bacterial surface and absent from the surface of the poles. In addition, the complexity of *L. plantarum* surface is evidenced by the fact that PG is accessible at the surface only in absence of WPS (Beaussart et al., 2013).

The structures of both *L. rhamnosus* and *L. plantarum* LTA were confirmed to be made of a poly(Gro-P) backbone with an average of 30 and 22 repeating units of Gro-P, respectively, (Grangette et al., 2005; Claes et al., 2012b). In both cases, D-Ala was found to be the unique detectable substituent. The lipid moiety of the *L. rhamnosus* LTA reveals an average fatty acid chain of C14 (Claes et al., 2012b). In *L. lactis*, poly(Gro-P) chains contained linked D-Ala and Gal (Giaouris et al., 2008; Kramer et al., 2008).

LTA AS BACTERIOPHAGE RECEPTORS IN LACTOBACILLI

A second model system where the bacteriophage receptors have been identified is the pair *Lactobacillus delbruekii* subsp. *lactis* ATCC15808 and bacteriophage LL-H. In this case, LTA were shown to be the phage receptor components (Raisanen et al., 2004). In addition, it was shown that D-Ala and α-Glc substituents of LTA affect the adsorption of LL-H phages. A high degree of D-alanylation decreased phage adsorption whereas Glc substituents were required for efficient binding (Raisanen et al., 2007). A model is proposed where the anti-receptor protein of the phage tail binds to the glucosyl- substituted glycerol of LTA, providing reversible, specificity-determining binding to the surface. Another domain of the antireceptor protein would ensure irreversible binding to the negatively charged

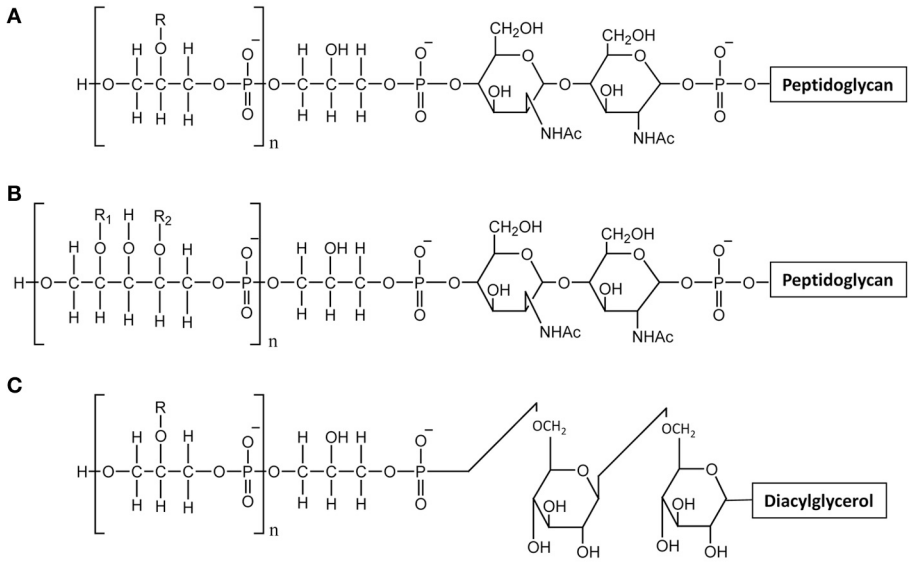

FIGURE 3 | Structure of teichoic acids. (A) WTA with poly-glycerol-phosphate chains; **(B)** WTA with poly-ribitol-phosphate chain; **(C)** LTA with poly-glycerol-phosphate chains. R, R1, R2 indicate potential substituent groups of polyols chains (e.g., D-Ala, Glc, Gal, GlcNAc).

poly-glycerol-phosphate chains (with no or low local level of D-Ala substituents) (Munsch-Alatossava and Alatossava, 2013).

PEPTIDOGLYCAN AS TARGET OF BACTERIOPHAGE ENDOLYSINS

PEPTIDOGLYCAN STRUCTURE IN LAB

PG is the most abundant polymer of the Gram-positive cell wall. It is composed of glycan strands, made of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetyl-muramic acid (MurNAc), which are cross-linked by short peptide chains (**Figure 1B**). Although the PG basic structure is characteristic for a given bacterial species (Schleifer and Kandler, 1972), PG is in a dynamic state throughout bacterial cell life, and its structure is the result of complex biosynthetic, maturation, and degradation reactions (Typas et al., 2012).

Structural analysis of the PG-constituting muropeptides of several LAB, such as *L. lactis* (Courtin et al., 2006), *L. casei* (Regulski et al., 2012), *L. rhamnosus* (Claes et al., 2012a), and *L. plantarum* (Bernard et al., 2011a) confirmed that the first three species have a D-Ala^4-D-Asp/Asn-L-Lys^3 cross-bridge whereas the latter has a direct D-Ala^4-mDAP^3 cross-bridge. Also, PG covalent modifications were revealed, including O-acetylation of MurNAc in the four species, O-acetylation of GlcNAc in *L. plantarum*, N-de acetylation of GlcNAc in *L. lactis*, amidation of D-Asp cross-bridge in *L. lactis*, *L. casei*, and *L. rhamnosus*, and amidation of mDAP in *L. plantarum*. O-acetylation of MurNAc is known to inhibit lysozyme (Bera et al., 2005) and all the PG modifications listed above were shown to control the activity of specific endogenous bacterial PGHs (named autolysins) (Veiga et al., 2009; Bernard et al., 2011a,b).

HYDROLYSIS OF PEPTIDOGLYCAN BY BACTERIOPHAGE ENDOLYSINS

Endolysins, encoded by phage DNA, are PGHs synthesized in phage-infected cells at the end of the multiplication cycle, and able to lyse bacteria and release phage progeny (Loessner, 2005). Endolysins usually lack a signal peptide for their export and therefore rely on the synthesis of holins which insert into the cytoplasmic membrane and make pores (**Figure 1A**) (Wang et al., 2000). Like bacterial PGHs, phage endolysins have a modular structure including a catalytic domain and a cell-wall binding domain (CWBD). Most often, their catalytic domain is located at the N-terminus and their CWBD at the C-terminus (Fischetti, 2008).

Generally, the catalytic domains found in endolysins belong to the same families as those encountered in bacterial PGHs (Chapot-Chartier, 2010). The different endolysins found in Siphoviridae phage genomes of *L. lactis* and different *Lactobacillus* species have been recently searched in available genome sequences (Oliveira et al., 2013) and are listed in **Table 1**. The catalytic domains found in these endolysins belong to five Pfam domain families which confer different hydrolytic specificities to the enzymes (**Figure 1B**). These domains include Amidase_2 domain (PF01510) conferring *N*-acetyl-muramyl-L-Ala-amidase activity, Glyco_hydro_25 (PF01183) conferring *N*-acetyl-muramidase activity, Phage_lysozyme domain (PF00959) conferring *N*-acetyl-muramidase activity, Amidase_5 (PF05382) conferring γ-D-Glu-L-Lys-endopeptidase activity (Regulski et al., 2013) and CHAP domain (cysteine, histidine-dependant amidohydrolase/peptidase domain) (PF05257) with both amidase and/or peptidase specificity (Frankel et al., 2011).

Interestingly, tail-associated lysins were also found in certain bacteriophages such as Tuc2009 and TP901-1. The tail fiber of these phages is composed of a trimer of Tal proteins which contain a PG-hydrolase domain of the M23-peptidase family (PF01551). This domain is protruding from the large host-recognizing baseplate structure of each of these phages (Kenny et al., 2004) and is most likely involved in PG digestion required for phage DNA injection inside the

Table 1 | Domain structure of the main endolysins of Siphoviridae phages infecting *L. lactis* and *Lactobacillus* species[a].

Name of the phage	Protein ID	Length (AA)	Catalytic domain		Cell wall binding domain
			Domain	Putative specificity	
Lactococcus lactis					
Phage SL4	ACU46783.1	234	Amidase_2 (PF01510)	Amidase	No
Phage CB13	ACU46835.1	234			
Phage P008	YP_762533.1	233			
Phage Q54	YP_762603.1	256			
Phage bIBB29	YP_002004009.1	233			
Phage bIL170	NP_047135.1	233			
Phage P087	YP_002875753.1	237			
Phage jj50	YP_764334.1	253	Amidase_2	Amidase	No
Phage 712	YP_764281.1	258			
Phage sk1	NP_044966.1	246			
Phage r1t	NP_695077.1	270	Amidase_2	Amidase	No
Phage 949	YP_004306215.1	343	Amidase_2	Amidase	Lc-LysBD[b]
Prophage bIL285	NP_076634.1	259	Amidase_5 (PF05382)	γ-D-Glu-L-Lys-Endopeptidase	PG_binding_3 (PF09374)
Prophage bIL286	NP_076695.1	259			
Prophage bIL309	NP_076751.1	259			
Phage BK5-T	NP_116519.1	259			
Phage bIL67	NP_042321.2	226	Phage_lysozyme (PF00959)	Muramidase	No
Phage c2	NP_043551.1	226			
Phage 4268	NP_839940.1	305	Glyco_hydro_25 (PF01183)	Muramidase	No
Phage phiLC3	NP_996722.1	429	Glyco_hydro_25		2 × LysM (PF01476)
Phage TP901-1	NP_112716.1	429			
Phage Tuc2009	NP_108734.1	428			
Phage ul36	NP_663692.1	429			
Phage P335	ABI54253.1	432			
Phage 1358	ADD25719.1	233	CHAP (PF05257)	Amidase or Endopeptidase	SH3_5 (PF08460)
Lactobacillus casei					
Prophage Lc-Lys Phage A2	YP_001987071.1 NP_680500.1	350	Amidase_2	Amidase	Lc-LysBD[b]
Prophage Lc-Lys2	YP_001986946	324	Amidase_5	γ-D-Glu-L-Lys-Endopeptidase	Lc-LysBD[b]
Phage phiAT3	YP_025045.1	393	Glyco_hydro_25	Muramidase	SH3_5, LysM
Lactobacillus rhamnosus					
Phage LC-Nu Phage Lrm1	YP_358779.1 YP_002117687.1	432	Glyco_hydro_25	Muramidase	2 × LysM

(Continued)

Table 1 | Continued

Name of the phage	Protein ID	Length (AA)	Catalytic domain		Cell wall binding domain
			Domain	Putative specificity	
Lactobacillus gasseri					
Prophage KC5a	YP_529896.1	246	Glyco_hydro_25	Muramidase	No
Phage phiadh	NP_050170.1	317	Glyco_hydro_25	Muramidase	SH3_5
Lactobacillus delbruekii* subsp. *lactis					
Phage LL-H	YP_001285906.1	298	Glyco_hydro_25	Muramidase	No
Lactobacillus delbruekii* subsp. *bulgaricus					
Phage c5	ACA63343.1	301	Glyco_hydro_25	Muramidase	SH3_5
Lactobacillus plantarum					
Phage LP65	YP_164723.1	464	Glyco_hydro_25	Muramidase	No
Phage phiJL-1	YP_223905.1	398	Glyco_hydro_25	Muramidase	SH3_5
Phage Sha1	ADW01314.1	390	Glyco_hydro_25	Muramidase	SH3_5 LysM
Phage phig1e	YP_003084340.1	442	Glyco_hydro_25	Muramidase	SH3_5 LysM
Lactobacillus johnsonii					
Prophage Lj928	NP_958555.1	315	Glyco_hydro_25	Muramidase	SH3_5

[a] *Data extracted from Oliveira et al. (2013).*

[b] *Lc-LysBD was characterized in Regulski et al. (2013).*

cytoplasm thus facilitating infection especially when PG is highly cross-linked. The hydrolytic specificity of the Tal PGH was shown to be a D-Ala-D-Asp/Asn endopeptidase allowing hydrolysis of PG peptide cross-bridges (**Figure 1B**), potentially making holes in the PG network (Stockdale et al., 2013).

PEPTIDOGLYCAN AS LIGAND OF BACTERIOPHAGE ENDOLYSIN CWBDs

The CWBD of bacteriophage endolysins is thought to maintain the proteins tethered to the cell wall after bacterial lysis. This will allow preventing further attack and lysis of adjacent bacterial cells that represent potential hosts for the new phage particles released upon lysis thus ensuring phage propagation. Very often endolysin CWBDs bind cell wall with high affinity and high specificity. Therefore, they were proposed for biotechnological applications such as identification of bacteria by specific staining (Schmelcher et al., 2010) or, after fusion with a protein of interest, for displaying this protein at the bacterial surface with potential applications such as vaccine or biocatalyst development (Lee et al., 2003; Visweswaran et al., 2014).

Lactococcus and *Lactobacillus* endolysins exhibit high diversity in their CWBD (Oliveira et al., 2013) and a number of them contain cell-wall binding modules commonly found in bacterial PGHs such as LysM or SH3b. However, a large number of endolysins do not display any sequence similarity in their C-terminal part with other known proteins and this C-terminal part could contain uncharacterized cell-wall binding modules.

The LysM module (PF01476) consists of a sequence motif of about 40 residues, which is widespread in eukaryotic and prokaryotic proteins, and often present as several repeats constituting a LysM-domain. It was found in several LAB PGHs such as the *L. lactis* major autolysin AcmA. LysM modules were shown to bind glycan chains of PG, involving most probably GlcNAc (Steen et al., 2003; Frankel and Schneewind, 2012).

The SH3 domain initially known in eukaryotes and virus was later on identified in bacterial PGHs. SH3 bacterial domains named SH3b (including different subfamilies SH3_3, SH3_4, and SH3_5) were reported to bind PG; however contradictory results were published regarding the exact recognized motif. It was concluded that the SH3-containing domain of ALE-1, an homolog of lysostaphin produced by *Staphylococcus simulans*, binds PG and that the length of the interpeptide cross-bridge and its amino acid composition have a major impact on the binding (Lu et al., 2006). Another study revealed that the C-terminal domain of lysostaphin which contain SH3_5

domain direct the enzyme to cross-linked PG (Grundling and Schneewind, 2006). In contrast, single molecule AFM experiments with tips functionalized with Acm2, the *L. plantarum* major autolyin containing five SH3_5 domains, concluded that SH3b domains rather bind PG glycan chains and involved GlcNAc (Beaussart et al., 2013).

Recently a CWBD, not described before, was characterized in the C-terminal part of prophage endolysins (Lc-Lys and Lc-Lys2) found in the complete genome sequence of *L. casei* BL23 (Regulski et al., 2013). This domain did not exhibit sequence identity with any known CWBD. It was demonstrated to bind PG and to be highly specific for amidated D-Asp cross-bridge present in *L. casei* PG (**Figure 1B**). It does not bind PG with another type of crossbridge such as L-Ala-L-Ala/L-Ser or even PG with non-amidated D-Asp cross-bridge. This domain (named Lc-LysBD) is also present in endolysins of other *L. casei* phages A2 and PL-1 as well as in *L. lactis* phage 949 endolysin (**Table 1**).

Another PG-binding domain (PG_binding_3 (PF09374)) is found in the C-terminal part of several endolysins listed in **Table 1**. However, the exact motif recognized by this domain is unknown.

CONCLUSIONS-PERSPECTIVES

The cell wall of LAB has received increased attention in the recent past years. Advances in structural studies of the cell wall and its components allow now the investigation of the molecular mechanisms of the interactions between bacteriophages and their host bacteria at several steps of the infection cycle. Further studies will aim at elucidating the inter-strain structural diversity of cell-wall polymers that are phage receptors at the bacterial surface, which could explain the narrow host range of certain *L. lactis* phages. Furthermore, the 3D-structures of several RBPs are available and the molecular determinants of the specificity of the binding of RBPs to the polysaccharide receptors can now be investigated. At the applied level, further knowledge will allow rational selection of LAB strains taking into account their WPS-types to design starters resistant to certain groups of bacteriophages with known RBPs or for strain rotation to prevent phage attack. Also, as already proposed previously with the use of camelid nanobodies raised against the purified baseplate complex (Desmyter et al., 2013), strategies based on the inhibition of the binding of RBP to their receptors may be considered at the molecular level on the basis of the 3D-structures of RBPs. In another field of applications, it is expected that new CWBDs could be discovered in phage-encoded endolysins and their ligands in the cell wall characterized. This improved knowledge will open new perspectives to construct tools to display proteins of interest at the bacterial surface of LAB for biotechnological applications.

ACKNOWLEDGMENTS

I thank Saulius Kulakauskas (Micalis, INRA, France) for valuable discussions and help in figure design. I thank all my present and past collaborators cited in this review. Work of MPCC was supported by Institut National de la Recherche Agronomique (INRA), Région Ile de France and French National Research Agency (ANR-11-BSV8-01 LactoPhages).

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 27 December 2013; accepted: 30 April 2014; published online: 22 May 2014. Citation: Chapot-Chartier M-P (2014) Interactions of the cell-wall glycopolymers of lactic acid bacteria with their bacteriophages. Front. Microbiol. **5**:236. doi: 10.3389/fmicb.2014.00236*

This article was submitted to Virology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 16 January 2014
doi: 10.3389/fmicb.2014.00003

Structures and host-adhesion mechanisms of lactococcal siphophages

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The Siphoviridae family of bacteriophages is the largest viral family on earth and comprises members infecting both bacteria and archaea. Lactococcal siphophages infect the Gram-positive bacterium *Lactococcus lactis*, which is widely used for industrial milk fermentation processes (e.g., cheese production). As a result, lactococcal phages have become one of the most thoroughly characterized class of phages from a genomic standpoint. They exhibit amazing and intriguing characteristics. First, each phage has a strict specificity toward a unique or a handful of *L. lactis* host strains. Second, most lactococcal phages possess a large organelle at their tail tip (termed the baseplate), bearing the receptor binding proteins (RBPs) and mediating host adsorption. The recent accumulation of structural and functional data revealed the modular structure of their building blocks, their different mechanisms of activation and the fine specificity of their RBPs. These results also illustrate similarities and differences between lactococcal Siphoviridae and Gram-negative infecting Myoviridae.

Keywords: bacteriophage, *Lactococcus lactis*, Siphoviridae, electron microscopy, crystal structure

INTRODUCTION

Lactococcus lactis is a Gram-positive bacterium extensively used for the production of fermented milk products, such as cheese production buttermilk and sour cream. *L. lactis*-containing starter cultures are therefore used world-wide in large scale industrial processes, which create ideal ecological niches for bacteriophage infections and development. These infections have a major economic impact due to impairment of lactococcal fermentations and the resulting need to close and decontaminate the production plants. Addressing this problem has proven to be challenging as *L. lactis* virulent phages are ubiquitous in plant environments and pasteurized milk (Moineau et al., 2002).

Hundreds of *L. lactis* phages strains have been isolated to date and they have been classified into 10 groups (Deveau et al., 2006). The lactococcal phage population is largely dominated by the Siphoviridae family, i.e., phages with a long non-contractile tail, with groups 936, P335 and c2 accounting for ∼80, 10, and 5% of these virions, respectively. Only two lactococcal phages from a different family have been isolated belonging to the short-tailed Podoviridae. No Myoviridae infecting *L. lactis* has been reported.

Phages from the c2 group have been shown to recognize and infect *L. lactis* using a protein receptor, the phage infection protein (PIP; Babu et al., 1995; Mooney et al., 2006). PIP is orthologous to the *Bacillus subtilis* protein YueB, a component of type VII secretion system (Abdallah et al., 2007) and the receptor of phage SPP1 (Sao-Jose et al., 2006; Vinga et al., 2012). In contrast, no proteinaceous receptor could be identified for other lactococcal phages, suggesting early on that they may use saccharidic receptors for infection (Valyasevi et al., 1990; Ruud et al., 1994; Deveau et al., 2002). A striking property of lactococcal phages is their narrow host specificity: each of the hundreds of lactococcal phages recognizes only one or a handful of *L. lactis* strains. This observation along with the absence of identified protein receptors supported the hypothesis that non-c2 phages use saccharidic receptors, since only polysaccharides could provide a sufficient diversity to rationalize this data.

This review focuses on the structure of lactococcal phages p2, a lytic phage, and TP901-1, a lysogenic phage, belonging to the predominant 936 and P335 groups, respectively. Their complete structures have been tackled using electron microscopy (EM), and the structure of their components involved in adhesion

was determined by X-ray crystallography. These structural data, together with functional studies, made it possible to reveal striking features of lactococcal phages concerning their baseplate activation, and the specificity of their receptor binding proteins (RBPs). Since the recent discovery of the *L. lactis* phospho-polysaccharide "pellicle," understanding lactococcal phages specificity at molecular levels begins to unravel (Andre et al., 2010; Chapot-Chartier et al., 2010).

OVERALL PHAGE STRUCTURE

Knowledge on phage structures has to date primarily relied on the structures of Myoviridae or Podoviridae, since the flexible tail of Siphoviridae has prevented the application of single-particle reconstruction in a straightforward manner (**Figures 1A,B**). Lactococcal phages TP901-1 and p2 EM structures could be determined by dissecting the phages in smaller parts: capsid, connector, tail segments, and tail tip. The EM single-particle reconstruction was performed individually and the structures of these parts were reassembled on a scaffold obtained from analysis of a few straight tailed phages (Bebeacua et al., 2013a,b; Sassi et al., 2013). Both phages possess a $T = 7$ *laevo* icosahedral capsid, and the major capsid protein (MCP) hexamer from phage HK97 (Wikoff et al., 2000) fits in the EM map with a satisfying correlation coefficient. No protruding decorations are present on the capsid surface in contrast with what has been reported for some other coliphages, such as T4 (Olson et al., 2001; Fokine et al., 2005). The connector structures of p2 and TP901-1 are similar to that of SPP1, comprising a dodecameric portal (Dube et al., 1993) and the two head-to-tail junction proteins (Lhuillier et al., 2009). The tail structures of phages TP901-1 and p2 are of comparable length, and the major tail protein (MTP) hexamers are of similar thickness, while their helical pitch is significantly different (Bebeacua et al., 2013a,b; **Figures 1C,D**). A striking difference between the two tails is the presence of decorations on the tail of phage p2. Sequence analysis of phage p2 MTP revealed that its N-terminus shares similarity with other MTPs from other phages, such as SPP1 and Lambda (Pell et al., 2009). At the C-terminus it possesses an adhesin fold which appears as decorations on the surface of the tail. In the cases of phages SPP1 (Auzat et al., 2008) and Lambda (Fraser et al., 2006; Pell et al., 2010), it has been suggested that such C-terminal domains help the primary adhesion of phages to their host. Such decorations have also been evidenced in the mycobacterial phage, Araucaria (Sassi et al., 2013). Large baseplate structures are present at the distal tail extremity, which can vary significantly in size and shape, and form the control center for infectivity (Bebeacua et al., 2010; Sciara et al., 2010). In contrast, phages Araucaria (Sassi et al., 2013), SPP1 (Plisson et al., 2007), T5 (Breyton et al., 2013), and Lambda (Davidson et al., 2012; Tam et al., 2013) exhibit a simplified tail tip, in agreement with the fact that these phages have been shown to recognize and attach to a host protein receptor.

GENOMIC COMPARISONS

Phages p2 and TP901-1 have similar structural genomic modules resembling that of phage SPP1 (Chai et al., 1993; Brondsted et al., 2001; Bebeacua et al., 2013b; **Figure 2**). A prominent feature of

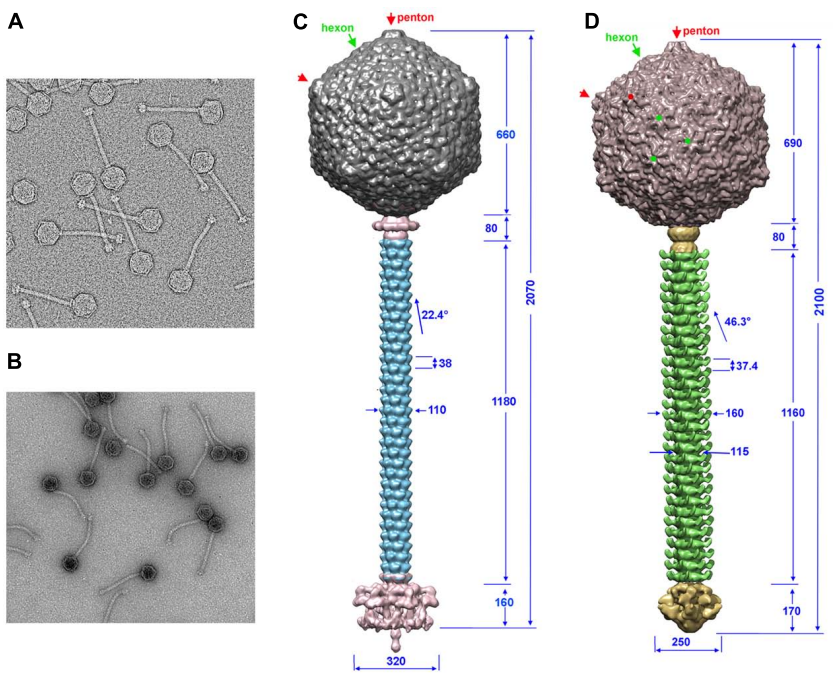

FIGURE 1 | The TP901-1 and p2 phages assembled structures. **(A,B)** Electron microscopy images of phages TP901-1 **(A)** and p2 **(B)**. **(C,D)** The structures of phage TP901-1 **(C)** and p2 **(D)** were generated by assembling the reconstructions of the capsid (top), connector and tail (middle), and the tail-tip (bottom) on low-resolution maps of the full phages. In the capsid map, pentons are identified by red arrows/points and hexons by green arrows/points. Dimensions are given in Å and the angle of rotation between MTP hexamers is given in degrees.

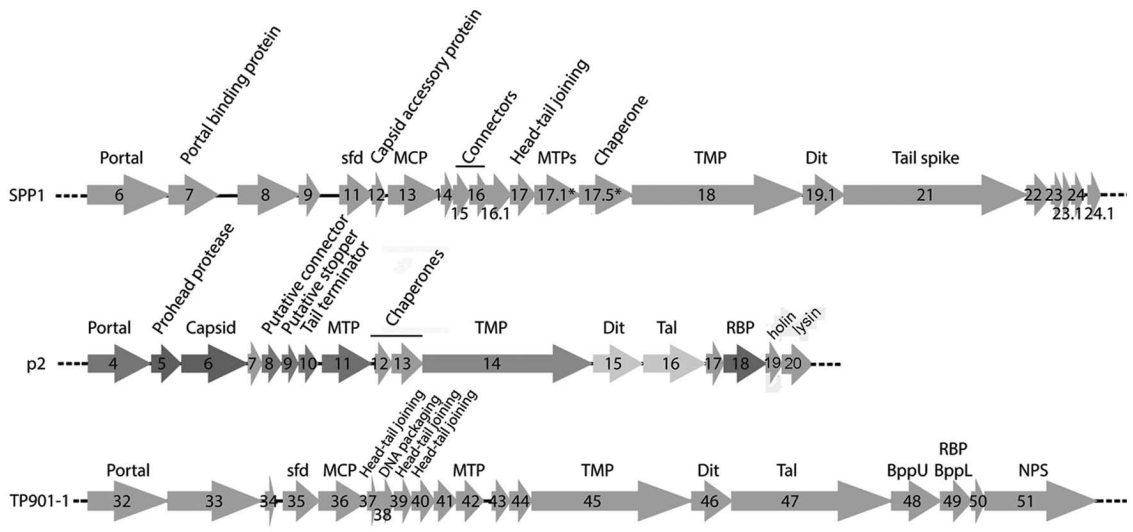

FIGURE 2 | Schematic representation and assignment of the structural gene module of phages SPP1, p2, and TP901-1. Genes coding for non-structural ORFs are in light gray. Sfd, scaffolding; MCP, major capsid protein; MTP, major tail protein; TMP, tail tape measure protein; Dit, distal tail protein; Tal, tail-associated lysine; RBP, receptor binding protein; BppU, baseplate upper protein; BppL, baseplate lower protein; NPS, neck passage structure. * indicates alternate splicing.

these modules is the long tape measure protein (TMP), which determines the length of the tail (Pedersen et al., 2000) and participates in the infection mechanism (Boulanger et al., 2008). Upstream, the genes of tail chaperones (Siponen et al., 2009a; Pell et al., 2013), MTP, capsid, and connector proteins are easily identified. Downstream, the first gene encountered is that of the distal tail protein (Dit) conserved in the three phages (Sciara et al., 2010; Veesler et al., 2010, 2012), but also in all Siphoviridae, including those infecting Gram-negative bacteria such as T5 (Flayhan et al., 2014). The X-ray structures of Dit have been determined and are presented below. Downstream the Dit *orf*, *Tal* genes exhibit varying lengths among phages, comprised between ∼400 and more than 1000 residues. HHpred (Soding et al., 2005) analysis revealed that the N-terminus (∼400 residues) of Tal proteins share a common fold, similar to that of T4 phage gp27 (Kanamaru et al., 2002), and to the type 6 secretion system (T6SS) VgrG module (Leiman et al., 2009). Downstream the Tal *orf*, large differences occur between the genes of the different phages in this region, reflecting the diverging structure of tail tips. In the case of phage p2, it has been demonstrated that the RBP open reading frame (ORF) is the last gene encoding for a structural protein and is located directly upstream the holin and lysin ORFs (**Figure 2**; Ledeboer et al., 2002; De Haard et al., 2005). Considering the sequence similarity between the p2 RBP (ORF18) and the TP901-1 RBP (ORF49), the same can be assumed for the latter phage. These two proteins were subjected to structural and biophysical studies.

While no peptidoglycan-digesting enzyme could be identified within the p2 structural cassette, the C-terminal moiety of the TP901-1 Tal has been shown to possess such an activity. Recent data demonstrated that TP901-1 mutated virions with Tal depleted of the peptidoglycan digesting enzyme domain could still infect their host during the *L. lactis* exponential phase growth, when the cell wall is either not or is less cross-linked to enable rapid cell division. In contrast, TP901-1 native phages possessing the peptidoglycan digesting Tal domain are able to infect the cell, even during the stationary phase (Stockdale et al., 2013).

RECEPTOR BINDING PROTEIN STRUCTURES

PHAGE p2

Llama immunization with p2 virions allowed to isolate single-domain llama antibody fragments (named VHH or nanobodies; Hamers-Casterman et al., 1993; Muyldermans et al., 2001) recognizing and neutralizing the p2 RBP. Such a nanobody (VHH5) was located at the distal part of the phage tail using immunogold labeling. Addition of this nanobody to a bacterial culture suppressed phage infection (Ledeboer et al., 2002; De Haard et al., 2005). Furthermore, it was demonstrated that VHH5 was an excellent binder of ORF18 (Kd value of ∼1.4 nM), identifying it as the RBP.

With in view to determine the receptor binding site of the p2 RBP, its crystal structure was determined alone and in complex with VHH5 (Spinelli et al., 2006b; Tremblay et al., 2006). The phage p2 RBP is an assembly of three chains of 264 amino acids forming a homotrimer. Noteworthy, a similar trimeric arrangement has also been observed in RBPs of mammalian adenoviruses and reoviruses (van Raaij et al., 1999; Chappell et al., 2002; Burmeister et al., 2004) as well as in the phage T4 gp12 protein (van Raaij et al., 2001). As observed in the phage T4 gp12 trimer (van Raaij et al., 2001), the p2 RBP is organized into three domains: shoulder, interlaced neck and head (**Figures 3A,B**). The shoulder domain (residues 1–141) has a β-sandwich fold assembling two 4-stranded anti-parallel β-sheets. A long helix contributed by each domain allows the three shoulder moieties to associate tightly. Immediately following the shoulder domains, the neck domain forms a triple-stranded β-helix of three segments organized into

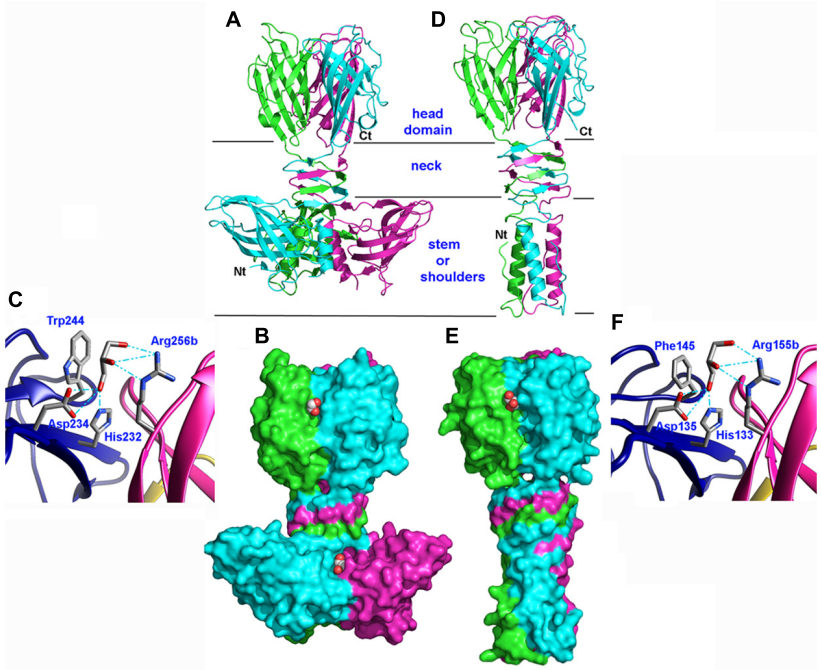

FIGURE 3 | Structures of the receptor binding proteins (RBPs) of phages p2 and TP901-1. (A,D) Ribbon view of the p2 RBP trimer **(A)** and of the TP901-1 RBP trimer **(D)**. Monomers are colored green, blue, and pink. **(B,E)** Surface representation of the p2 RBP trimer **(B)** and of the TP901-1 RBP trimer **(E)**. Bound glycerol molecules are represented by spheres (carbon, white; oxygen, red). **(C,F)** Close-up view of glycerol in the receptor binding site of the RBPs of phages p2 **(C)** and TP901-1 **(F)**. The glycerol molecule and the side-chains of the residues participating to binding are represented as sticks (carbon, white; oxygen, red; nitrogen, blue).

four β-strands along the threefold symmetry axis (β-prism). This very rigid neck structure has already been observed in the gp12 short tail fiber of *Escherichia coli* phage T4 (Myoviridae family), in which it plays a similar role, linking the N-terminal domain and the receptor binding head (van Raaij et al., 2001). The phage T4 puncturing device also contains a similar structure, but of a much larger diameter (Kanamaru et al., 2002). The RBP head domain follows the neck and forms a β-barrel comprising seven anti-parallel β-strands. Each β-barrel in the trimer is parallel to the threefold axis and interacts with the other two, yielding a very compact structure (**Figures 3A,B**). It exhibits low but significant similarity to RBPs of other viruses: the reovirus attachment protein σ1 trimer (Chappell et al., 2002) and the head domain of the adenovirus fiber (van Raaij et al., 1999).

PHAGE TP901-1

The phage TP901-1 RBP trimer structure was determined by X-ray diffraction (Spinelli et al., 2006a; Bebeacua et al., 2010). Its N-terminal segment is composed of elongated chains (residues 1–10), β-turns, and a three-helix-bundle assembled through non-polar side chain contacts (**Figures 3D,E**). This "stem" domain is much smaller than the corresponding shoulder domain in the phage p2 RBP (**Figures 3A,B**). However, in the p2 RBP, the three parallel helices (residues 19–32) located close to the threefold axis, are in a similar location to those of the TP901-1 RBP (**Figures 3A,D**).

Following the helix bundle domain, a short linker structure (residues 31–39) connects the α-helical domain (17–30) and the β-prism (40–63; **Figures 3D,E**). The β-prism neck domain interlaces three segments of each subunit, and each of its three faces is made of four β-strands from the three monomers, constituting a domain comparable to that of the RBP of phage p2 (Spinelli et al., 2006b) and to a streptococcal lyase (Smith et al., 2005). The amino acid sequence of the TP901-1 RBP neck region exhibits a six residue long, regular and repeating motif, not observed in phage T4 gp12 nor in the RBP neck of phage p2. Each segment ends with a polar residue, except the last one where it is replaced by a proline, which redirects the peptide chain upward, similar to the p2 RBP (Spinelli et al., 2006b).

The RBP head domain of TP901-1 (residues 64–163; **Figure 3D**) is a β-barrel formed of anti-parallel β-strands. This domain is the only part that shares sequence similarity with the RBP of p2 (28% sequence identity), and they exhibit very similar structures (**Figures 3A,D**). Noteworthy, the crystal structure of the RBP head domain from phage bIL170 (936 group) displays the same fold as in phages p2 and TP901-1 (Ricagno et al., 2006). The modular nature and interchangeability of RBP domains has been demonstrated by producing a chimeric RBP in which the N-terminal and linker RBP domains (stem and neck) of phage TP901-1 were fused to the C-terminal RBP head domain of phage p2 (Siponen et al., 2009b). The structure of this chimera has been determined by X-ray crystallography and it exhibits a stable conformation that closely resembles the parental structures, while a slight displacement of the linker improves the domains junction. Indeed, the receptor-binding site is structurally indistinguishable

from that of native p2 RBP and the chimera binds glycerol with equal affinity (see below).

THE RECEPTOR-BINDING SITE

PHAGE p2

The high-resolution structure of the p2 RBP (1.7 Å resolution) revealed the presence of three glycerol molecules (originating from the cryoprotectant liquor) bound at the interface between the head domains (Tremblay et al., 2006). The glycerol molecules are tightly bound (B-factors of 17.8 $Å^2$) via three hydrogen bonds established between the His 232 and Asp 234 side-chains and the glycerol O1 atom, and between the Arg 256 (from the other monomer) guanidyl group and the glycerol O2 atom (**Figure 3C**). Furthermore, the hydrophobic face of glycerol packs against Trp 244 side-chain, as often observed with sugar complexes (Bourne et al., 1990). Considering the close vicinity of Trp residues to glycerol molecules (Trp 244 and Trp 43), fluorescence quenching experiments made it possible to measure the affinity of the RBP glycerol binding site with glycerol and four different saccharides, and their Kd values ranged from 0.26 to 0.13 μM (Tremblay et al., 2006).

The structure of the complex between p2 RBP and VHH5 was also determined by X-ray crystallography and revealed that the nanobody covers a large area of the head domain (Tremblay et al., 2006; **Figures 4A,B**). A specific interaction is observed between Tyr 55 of the nanobody, penetrating deeply in the glycerol-binding site (**Figure 4C**), at the exact position occupied by the latter in the isolated RBP structure, and establishing interactions with Trp 244a and Arg 256b on either side. The OH group from Tyr 55 superimposes with the OH1 of glycerol and establishes similar hydrogen bonds with His 232 and Asp 234.

PHAGE TP901-1

Glycerol molecules were also observed bound at the interface between head domains in the crystal structure of the phage TP901-1 RBP at 1.6 Å resolution (Spinelli et al., 2006a). Glycerol molecules (**Figure 3F**) are stacked against Phe 145 and establish hydrogen bonds with His 133, Asp 135, and Arg 155. As inferred from sequence alignments, three of these residues in the TP901 RBP are identical to those in the RBP of phage p2, while the fourth one corresponds to a substitution of Phe 145 by Trp 244. Two hydroxyl

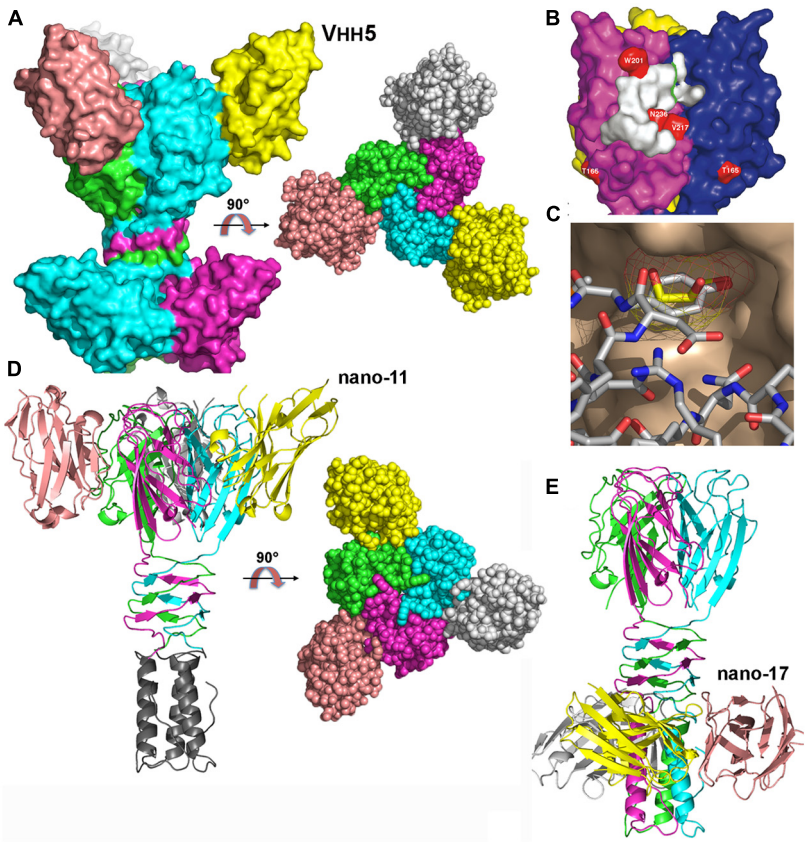

FIGURE 4 | Structures of the receptor binding proteins (RBPs) of phages p2 and TP901-1 in complex with VHHs/nanobodies. **(A)** Surface representation of the p2 RBP trimer in complex with the neutralizing VHH5 (nano5), and 90° rotated view. **(B)** Surface footprint of VHH5 on the RBP trimer surface (white). Mutated residues leading to neutralization escape are indicated in red. **(C)** View of the superposition of the VHH5 Tyr 55 with glycerol. The RBP surface is colored beige, the glycerol carbon atoms are yellow, while those of VHH5 are white. Oxygen atoms are red and nitrogen atoms are blue. **(D)** Ribbon view of the TP901-1 RBP trimer in complex with the neutralizing nanobody 11 and surface view at 90° (right). **(E)** Ribbon view of the TP901-1 RBP trimer in complex with the non-neutralizing nanobody 17. Panels **(B,C)** taken from Tremblay et al. (2006).

groups from the glycerol molecule are therefore strongly bound to the RBP head. In contrast, the third hydroxyl group is free and points to the bulk solvent in both structures (**Figure 3F**). This orientation strongly suggests that the saccharidic binding site harbors the terminal residue of the receptor polymer, the free hydroxyl group pointing in the direction of the rest of the receptor polymer attached to the host.

The superposition of the binding network of glycerol in both RBPs shows their striking similarity (**Figures 3C,F**). Differences in amino acid residues are only observed in the second binding sphere, which modulates the binding crevice surface and volume and, hence, may influence the saccharidic specificity observed between different phages and bacterial strains. The absence of tryptophan residue in the vicinity of the glycerol-binding site in the phage TP901-1 RBP prevented us to directly perform binding studies using tryptophan fluorescence quenching. A Phe145Trp point mutant was therefore designed and characterized the affinity of the complexes formed with glycerol, phospho-glycerol, *N*-acetyl muramic acid, muramyl-dipeptide, and galactose. The obtained affinity constants were roughly comparable to those measured for the p2 RBP (Spinelli et al., 2006a).

The structures of the TP901-1 RBP in complex with nanobodies (Desmyter et al., 2013) and designed ankyrin repeat proteins (DARPins; Veesler et al., 2009) were also obtained. In the first case, the, TP901-1 baseplate (see below) was used for llama immunization. Among the different binders characterized, three of them targeted the RBP: nanobodies 2, 11, and 17. Functional studies demonstrated that nanobodies 2 and 11 could neutralize phage infection, while nanobody 17 could not (Desmyter et al., 2013). Indeed, the binding sites of nanobodies 2 and 11 were localized at the interface between head domains, occupying the glycerol binding site (**Figure 4D**). In contrast, nanobody 17 binds the stem domain and does not interfere with the receptor binding site (**Figure 4E**; Desmyter et al., 2013). In the second case, DARPin binders were generated against a subcomplex of the TP901-1 baseplate (i.e., the BppU/RBP tripod complex). We isolated three binders and demonstrated they targeted the RBP and in turn neutralized phage infection (Veesler et al., 2009). The structure of one of them in complex with RBP was obtained revealing a totally different binding mode compared to the VHH/nanobody complexes. A unique DARPin binds at the tip of the RBP head domain, leaving the receptor binding site free, but probably blocking the direct interaction with the host (see below the baseplate section; Veesler et al., 2009).

THE SACCHARIDIC RECEPTORS AND THEIR INTERACTIONS WITH THE RBPs

The affinity of glycerol and phospho-glycerol for the RBPs suggested that lipoteichoic acids (LTA) could act as receptors for lactococcal phages. Conversely, the observation that many sugars bind equally well to the RBPs and the fact that the structure of LTAs is too simple to explain the different specificities of hundreds of lactoccocal phages constituted arguments against this hypothesis. A recent report by Chapot-Chartier et al. (2010) revealed that the surface of the *L. lactis* cell wall is covered by a "pellicle," constituted of hexasaccharide phosphate repeating units that are distinct from any other bacterial polysaccharides,

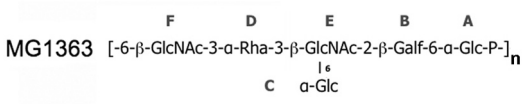

FIGURE 5 | The "pellicle" phospho-polysaccharide from *L. lactis* MG1363 (Chapot-Chartier et al., 2010). This phospho-polysaccharide is the receptor of lactococcal phages sk1 and p2.

which appears as a strong candidate to allow phage adsorption (**Figure 5**). Indeed, *L. lactis* mutants lacking this "pellicle" could not be infected by their specific phage and genomic studies demonstrated the presence of *L. lactis* strain-specific "pellicle" cassettes coding for glycosyl-transferases and other enzymes involved in polysaccharide phosphate synthesis (Mahony et al., 2013). Finally, the diversity induced by ∼6-mer saccharides is fully compatible with the fine specificity of lactococcal phages.

Preliminary experiments using surface plasmon resonance (SPR) explained the specificity mechanism of phage p2 RBP for *L. lactis* strain MG1363 (Bebeacua et al., 2013b). The purified pellicle of this *L. lactis* strain was biotinylated and attached to a SPR chip (ligand) while either the RBP of phage p2 or of phage TP901-1 was injected (analyte) to monitor interactions. While a typical saturation curve was obtained for the p2 RBP, yielding a Kd value of 230 ± 40 nM, it was not possible to reach saturation with the TP901-1 RBP. More significantly, the dissociation time of the TP901-1 RBP with the *L. lactis* MG1363 pellicle is extremely short as compared to that of the p2 RBP. As a result, phage TP901-1 would remain in contact for a very short time with the specific host of phage p2, which would be insufficient for adhesion and further infection (Bebeacua et al., 2013b).

BASEPLATE STRUCTURES AND MECHANISMS OF ACTIVATION

THE PHAGE p2 BASEPLATE

Based on their genomic location, we hypothesized that *orfs* 15 (Dit), 16 (Tal), 17, and 18 (RBP) encoded baseplate-related proteins (**Table 1**). The contiguous cluster of four genes was cloned, expressed in *E. coli* and purified, yielding a macromolecular complex of ∼1.0 MDa containing ORFs 15, 16, and 18 (Campanacci et al., 2010). ORF17 could not be detected in this assembly, in agreement with its absence in mature virions. Although crystals of the complex were obtained readily, they did not diffract beyond 8 Å resolution. In contrast, after mixing with an excess of VHH5, a new complex was obtained that crystallized and diffracted to 2.6 Å resolution (Sciara et al., 2010). The baseplate-VHH5 (BP-VHH) structure is 230 Å wide and 160 Å high, displays a quasi hexagonal symmetry, and is formed from bottom to top by three ORF16, six ORF15, and six trimers of ORF18, as well as 18 VHH5 (**Figures 6A,B**).

ORF15 (Dit) is composed of two domains. The N-terminal domain ("ring domain" 1–132) shows a split barrel-like fold similar to that found in phage Lambda gpV (Pell et al., 2009) and Hcp, a T6SS protein (Jobichen et al., 2010; Veesler and Cambillau, 2011). A long kinked extension (the "belt") of four β-strands embraces the next ORF15 molecule in the hexameric ring (**Figure 7A**). The

Table 1 | List of the components of the baseplates from lactococcal phages mentioned in this review.

Protein\phage	Abbreviations	p2	TP901-1	Tuc2009
Tape measure protein	TMP	ORF14	ORF45	ORF48
Distal tail protein	Dit	ORF15	ORF46	ORF49
Tail associated lysozyme	Tal	ORF16	ORF47	ORF50
Baseplate protein (upper)	BppU	n/a	ORF48	ORF51
Baseplate protein A	BppA	n/a	n/a	ORF52
Receptor binding proteinBaseplate protein (lower)	RBP(BppL)	ORF18	ORF49	ORF53

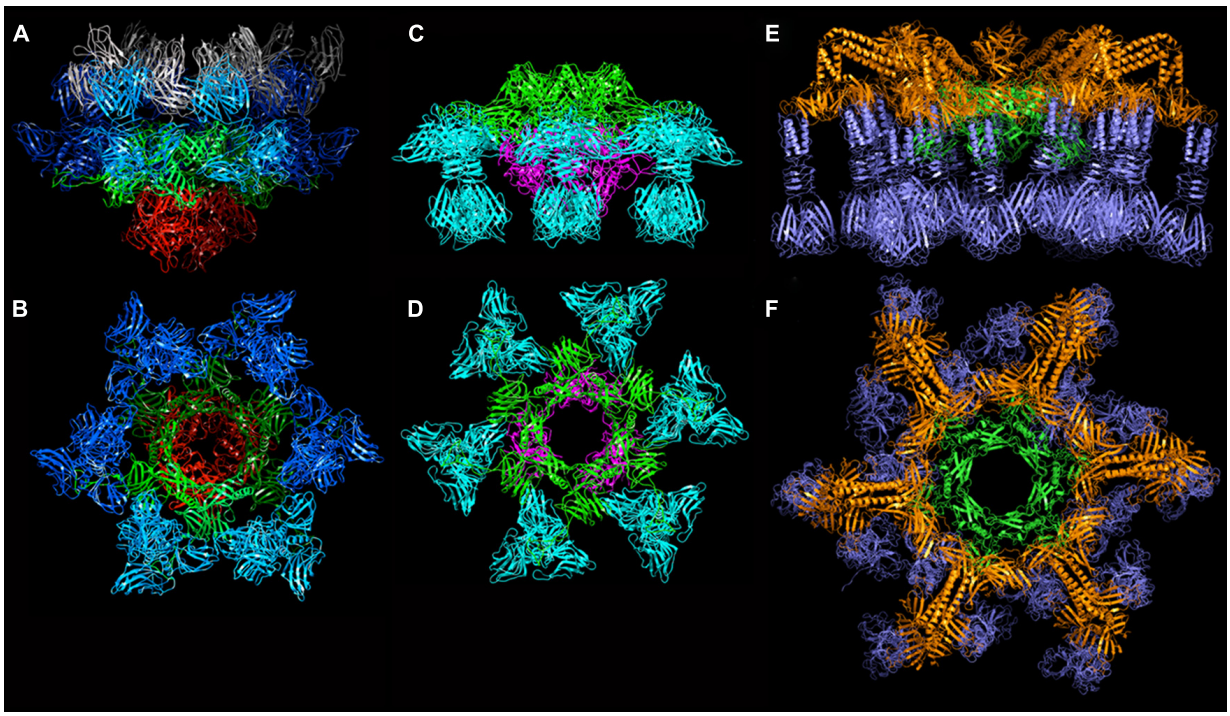

FIGURE 6 | The crystal structures of the baseplates of phages p2 and TP901-1. (A) Side-view of the phage p2 baseplate rest form in complex with the llama VHH5 (ORF15/Dit, green; ORF16/Tal, red; ORF18/RBP, blue; VHH5, gray). **(B)** Top-view of the phage p2 baseplate rest form (same colors are in **A**, but the VHH5 has been removed from the view). **(C)** Side-view of the phage p2 baseplate Sr^{2+}/Ca^{2+} activated form (same colors as in **A**). **(D)** Top-view of the phage p2 baseplate Sr^{2+}/Ca^{2+} activated form. **(E)** Side-view of the phage TP901-1 baseplate (ORF46/Dit, green; ORF48/BppU, orange; ORF49/RBP, violet). **(F)** Side-view of the phage TP901-1 baseplate (same colors as in **E**).

N-terminal domains form a tight ring with two layers of β-strands. This ring delineates a 40 Å-wide channel to allow the transit of the dsDNA genome during infection. The C-terminal domains (residues 137–275) are located at the ring periphery, and do not contact each other (**Figure 7A**). They display a galectin fold supplemented by a long extension (the "arm," residues 147–188) having a critical role in baseplate assembly via the formation of a three-digit hand that anchors the N-terminal domain of the RBP (ORF18, see below; **Figure 7A**).

ORF16 (Tal) is a 398 residue-long protein harboring four domains (**Figures 7B,C**) and its fold is similar to gp27 of myophage T4 (Kanamaru et al., 2002). In contrast to gp27, the ORF16 trimer forms a dome at the distal extremity of the baseplate, thereby closing its central channel (**Figures 6B** and **7C**).

The structure of ORF18 (RBP) is similar to that of ORF18 crystallized alone, with one exception: the N-terminal residues 2–17 of ORF18 in the baseplate structure are ordered and visible in the electron density. This is due to a tight interaction with the three-digit hand from the ORF15 galectin domain (**Figure 7A**). Furthermore, residues 2–7 of ORF18 protrude from each subunit, forming the first strand of the shoulder domain of the next subunit by domain-swapping (Sciara et al., 2010). As with the isolated protein, each ORF18 trimer is coordinated by three VHH5. The 18 VHH5 molecules together with the head domains of ORF18 build a large complex assembled through tight protein–protein contacts that stabilize the ORF18 position and that likely led to better diffracting crystals. Each ORF16 contacts two ORF15, which in turn attach two ORF18 trimers. There are no contacts between

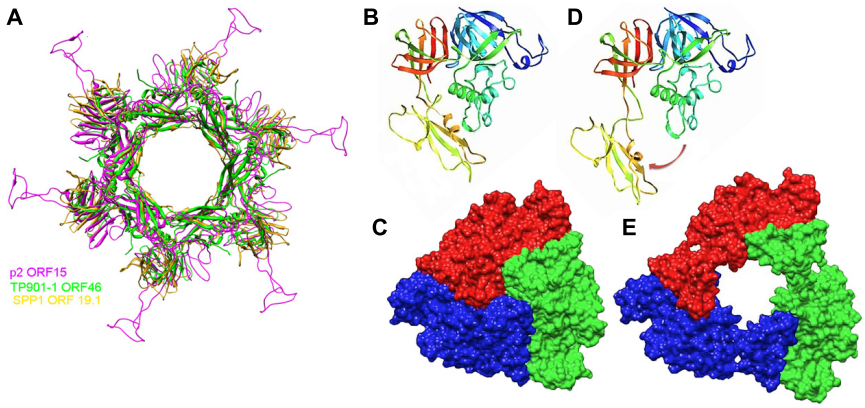

FIGURE 7 | Crystal structures of components of phages baseplates. **(A)** Superimposition of the Dit hexamers structures from phages TP901-1 (ORF46, green), p2 (ORF15, purple), and SPP1 (gp19.1, gold). **(B)** Ribbon view of the crystal structure of ORF16/Tal from the p2 baseplate in the rest form (rainbow coloring, from blue to red). **(C)** Surface view of the closed ORF16/Tal trimer from the p2 baseplate in the rest form. **(D)** Crystal structure of ORF16/Tal from the p2 baseplate in the Sr^{2+}/Ca^{2+} activated form. Domain 4 has moved away from the rest of the molecule. **(E)** Surface view of the open ORF16/Tal trimer from the p2 baseplate in the Sr^{2+}/Ca^{2+} activated form.

ORF16 and ORF18, and therefore, ORF15 hexamer plays the role of a central hub to which ORF16 and ORF18 are attached (**Figures 6A,B**).

The structure of the baseplate reported above exhibited an unexpected conformation. Indeed, one would have expected the head domains of the RBPs (ORF18), which harbor the receptor-binding sites, to point "downward," i.e., in the direction of the host cell surface. Instead, the RBPs were observed in a "heads-up" conformation, a position not compatible with optimal adhesion. However, the baseplate crystal structure fitted without rearrangement in the baseplate region of the p2 virion reconstruction (Sciara et al., 2010).

Since it was noticed that in some cases lactococcal phages infection required Ca^{2+}, attempts were made to obtain crystals in the presence of Ca^{2+} or Sr^{2+} without VHH5. New crystal forms were obtained readily with both cations, and their structure determined. Both structures were found to be identical, although the Sr^{2+} complex diffracted to higher resolution (Sciara et al., 2010). The complex comprised six ORF15, three ORF16, and six ORF18 trimers (**Figures 6C,D**). The most striking feature was that the RBPs have rotated by ∼200°, to orient their receptor-binding sites toward the distal phage extremity, leading to a "heads down" conformation (**Figures 8** and **9A,B**). The ring formed by the N-terminal domain of ORF15 superposed well between the Sr^{2+}-free and Sr^{2+}-bound structures, but the galectin, arm and hand domains had moved significantly (Sciara et al., 2010). The Sr^{2+} ion (or Ca^{2+} ion) is located at the interface between the N-terminal and the galectin domains of ORF15, and is coordinated by side chains of residues Asn10, Asp12, Asn241, Asp246, and the main-chain carbonyl of Leu11 (Sciara et al., 2010). The arm domains have rotated so that they are oriented in opposite direction compared to the "heads-up" structure. The ORF16 trimer was strongly affected, resulting in the opening of the dome with the concomitant formation of a channel of ∼32 Å diameter (**Figures 6D, 7D,E,** and **9A,B**), large enough for dsDNA passage. This opening results from a outward rotation of ORF16 cores with respect to the channel axis, and the opening of a crevice between domains 1, 2, 4, and 3 in an iris-like movement (**Figures 7D,E**). Domain 3 remains in close contact with the next ORF16 in the trimer (**Figure 7E**). In the activated baseplate structure, extensive interactions are established between ORF16s and ORF18s whereas these protein components were not in contact in the BP-VHH structure. In fact, these contacts lock the ORF18s in their "heads-down" conformation, giving to ORF16 the role played by the VHH5 molecules in the BP-VHH complex. Remarkably, the head domains are also maintained by the arm extensions belonging to the proximal Dit domains in the virion before being released when activation occurs (**Figure 8**).

THE PHAGE TP901-1 BASEPLATE

Following the same strategy employed for phage p2, attempts were made to express the phage TP901-1 baseplate by cloning a segment encompassing the *orfs* located between the *dit* and the *rbp* genes (*orfs* 46–49). Although this strategy was unsuccessful, a complex comprising only ORFs 46, 48, and 49 (without Tal, ORF47), could be expressed and purified, and its crystal structure determined at 3.8 Å resolution (Campanacci et al., 2010; Shepherd et al., 2011; Veesler et al., 2012). The TP901-1 baseplate is 320 Å wide and 160 Å high, exhibiting an overall sixfold symmetry, and a mass of 1.76 MDa (**Figures 6E,F**). From the proximal to distal end, it is formed by a Dit hexamer (ORF46) surrounded by 18 copies of BppU (ORF48) holding 54 RBPs (ORF49) organized as 18 trimers (**Figures 10A–D**). All together, it forms a complex of 78 proteins and 54 possible receptor binding sites, organized in six tripods each containing three BppU and three trimeric RBPs (**Figure 9C**; Veesler et al., 2012). Noteworthy, immunization of llamas with this baseplate complex led to tens of nanobody binders, among which only two proved to definitively neutralize phage infection (Desmyter et al., 2013).

The Dit forms a hexameric circular-shaped core with a 80 Å diameter, which delineates a 37 Å wide central channel (**Figures 6E,F**). Six domains are appended to this core without

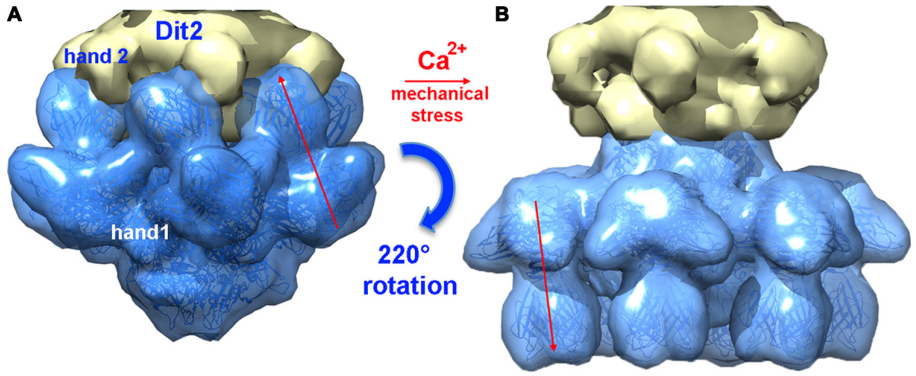

FIGURE 8 | A composite X-ray/EM reconstruction of the p2 baseplate. (A) A 20 Å electron density map (blue; ribbon structure inside) of the rest form (free virion) of the p2 baseplate crystal structure was calculated and subtracted from the baseplate experimental EM map. The resulting difference map (yellow) corresponds to a Dit (ORF15) hexamer. **(B)** A 20 Å electron density map (blue; ribbon structure inside) of the activated form of the p2 baseplate crystal structure was calculated and appended to the upper Dit EM map (yellow). Figure adapted from Bebeacua et al. (2013b). Copyright © American Society for Microbiology.

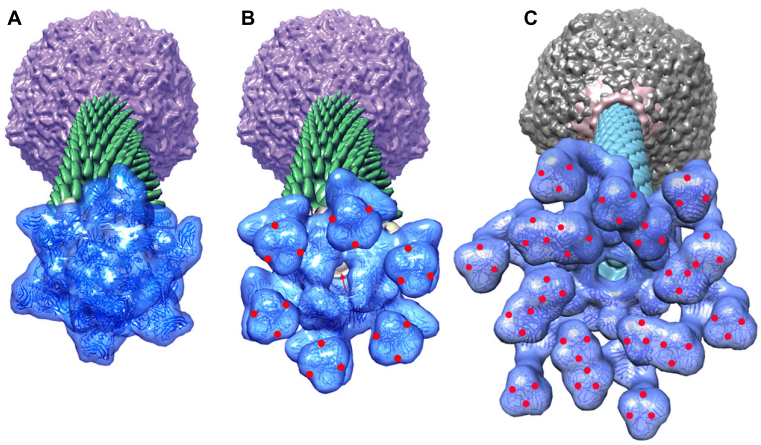

FIGURE 9 | Perspective views of the reconstructions of the p2 phage. (A) The baseplate rest form and **(B)** the Ca^{++} activated form, showing in forefront the baseplate structure, closed and opened, respectively. **(C)** The TP901-1 baseplate. The red dots are located at the RBP saccharides binding sites in the activated phage p2 and in the phage TP901-1 representations. Red arrow identifies the open channel of phage p2 activated baseplate. Panels **(A,B)** adapted from Bebeacua et al. (2013b). Copyright © American Society for Microbiology.

forming contact with each other (**Figure 7A**). Each monomer is formed from a N-terminal domain (residues 1–145) with a β-sandwich, an α-helix, and a β-hairpin, followed by a C-terminal domain (residues 146–255) folded as a galectin-like β-sandwich (Sciara et al., 2010; Veesler et al., 2010; **Figure 7A**). This Dit structure is similar to that of phages SPP1 (Veesler et al., 2010) and p2 (Sciara et al., 2010), demonstrating that this module forms the adsorption apparatus hub in phages of Gram-positive bacteria (Veesler and Cambillau, 2011) and beyond (Flayhan et al., 2014).

The 18 BppU assemble as six asymmetric trimers connecting the Dit central core and the RBPs (**Figures 6E,F** and **10A,B**). Each monomer is composed of a N-terminal globular domain (1–122; **Figure 10C**), a linker (123–134), two helices joined by a kink (135/139–194) and a globular C-terminal domain (195–299; Veesler et al., 2012). The C-terminal domains fold as β-sandwiches and assemble as a threefold symmetric triangular-shaped trimer held via two types of antiparallel pairing (**Figure 10D**). This structure binds to the three stem domains of three RBP trimers (**Figure 6F**). Each BppU C-terminal domain deeply projects a loop (residues 217–234) in the crevice formed at the top of the RBP trimer to anchor it to the baseplate via electrostatic interactions (**Figure 10E**). Moreover, three aliphatic/aromatic residues belonging to BppU (Ile 219, Phe 226, and Phe 232) fill the center of the RBP crevice. The conservation of the residues involved in the BppU/RBP interactions suggests that common architectural themes are found among P335-phages (Veesler et al., 2012).

The RBP structure is identical to the structure of its isolated form, with the three domains forming a trimer (Bebeacua et al., 2010). The three RBPs within each tripod are separated by at least 20 Å whereas extensive inter-tripod contacts involving the 12 most

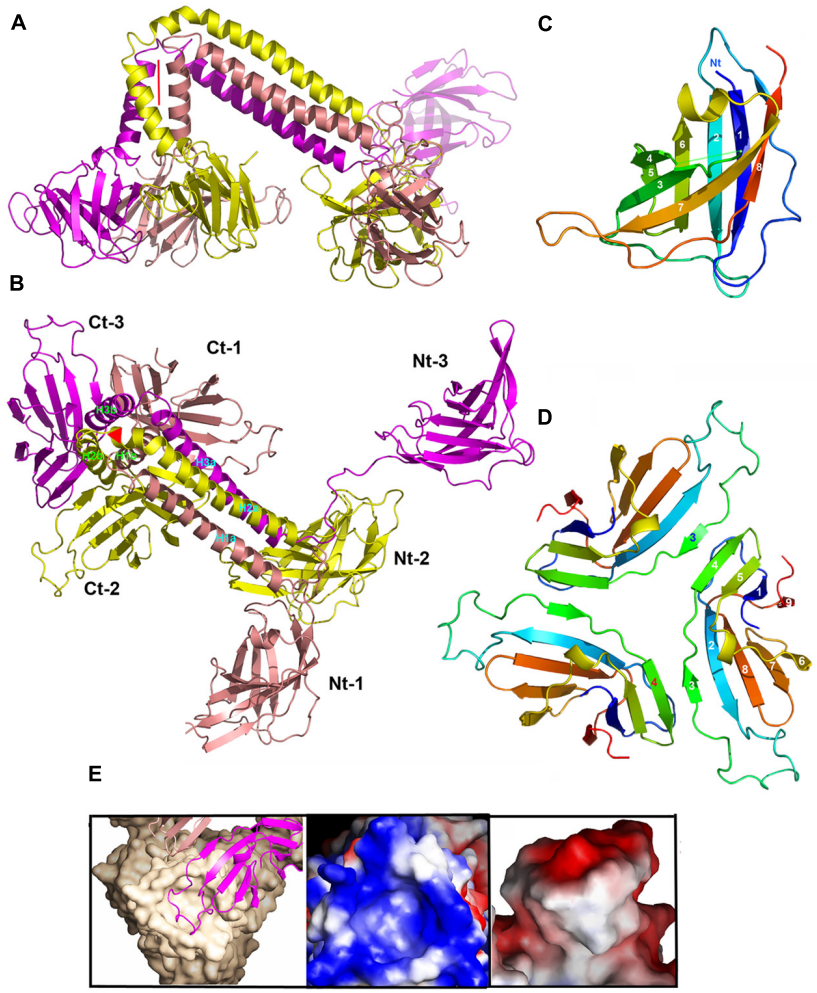

FIGURE 10 | Structures of components of phage TP901-1 baseplate. **(A)** Lateral ribbon view of the ORF48 trimer (salmon, yellow, and violet, for monomers 1, 2, and 3, respectively). **(B)** View 90° from **(A)** (top view) of ORF48 trimer. The N- and C-terminal domains are labeled, 1, 2, 3, respectively. **(C)** The ribbon view (rainbow colored) of the N-terminal domain of ORF48. **(D)** The ribbon view (rainbow colored) of a trimer of the C-terminal domain of ORF48. **(E)** Left to right: Close-up view of the electrostatic surface potential of the interacting regions from BppU and the RBP highlighting their high charge and surface complementarity. Each RBP trimer (beige) is anchored to the baseplate via a loop extending from each BppU C-terminal domain (pink) that penetrates the cup formed at the top of this former protein.

internal RBPs are observed (**Figure 6F**). The six most peripheral RBPs do not establish any contact and appear to be highly mobile (Bebeacua et al., 2010; Veesler et al., 2012).

This baseplate structure is most probably shared, more or less closely, by several phages from the P355 group and beyond. EM studies have demonstrated the structural resemblance of the P335 phage Tuc2009 baseplate with that of TP901-1 (Sciara et al., 2008; Collins et al., 2013). The major difference between these two baseplates is the presence of an extra protein termed BppA in the Tuc2009 baseplate whose gene is located between *bppU* and *bppL*. It was shown that this protein increases the binding specificity and/or affinity of the Tuc2009 tripod to its host receptor (Collins et al., 2013). Although the overall sequence identity between Tuc2009 and TP901-1 phage genomes is higher than 95%, the RBPs differ significantly: the stem and neck domains display high sequence identity, while the head domain displays no identity at all. Indeed, both phages target different *L. lactis* strains (UC509.9 and 3107, respectively), which likely harbor different pellicles in terms of composition and structure.

ADHESION MECHANISMS OF LACTOCOCCAL PHAGES

Comparison of the structures of the p2 and TP901-1 baseplates revealed that the latter is already in a "ready to adsorb" conformation without requiring any conformational change. This observation could be correlated with functional data as p2-like phages are non-infectious in the absence of Ca^{2+} whereas TP901-1-like phages do not require Ca^{2+} for infection (Veesler et al., 2012). These results could be explained by the presence of a conserved Ca^{2+}-binding loop in the Dit of p2-like phages that is absent in TP901-1-like phages, allowing to rationalize the different activation mechanisms exhibited by these different lactococcal phage

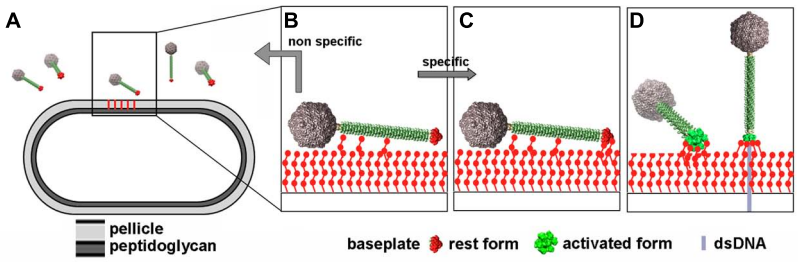

FIGURE 11 | Putative infection mechanism of *L. lactis* MG1363 by phage p2. (A) The phages in the vicinity of the host. **(B)** Weak interactions are established between the tail adhesins and putatively the pellicle. **(C)** Strain-specific lateral interactions may occur between the phage RBPs of the resting baseplate and the specific pellicle, leading, in the presence of Ca^{++} to **(D)** baseplate activation, RBPs rotation, and strong binding involving several of the 18 saccharide binding sites. Figure taken from Bebeacua et al. (2013b).

families. Furthermore, before specific interactions elicited by the baseplate/pellicle binding occur, a specific binding should maintain the phage long enough in the vicinity of its host in order to scout the cell wall for the proper receptor. In siphophages SPP1 (Auzat et al., 2008) and Lambda (Fraser et al., 2006; Pell et al., 2010) affinity modules have been described in the tail that exert this role. Whereas phage TP901-1 is devoid of these modules, phage p2 possess a tail decorated with such modules (Bebeacua et al., 2013b). This observation leads to suggest a more complete and realistic mechanism for phage p2 adhesion to its host (**Figure 11**).

CONCLUDING REMARKS AND PERSPECTIVES

The fine specificity of lactococcal phages for their *L. lactis* host strains can now be rationalized considering the diversity allowed by the chemical structure of the "pellicle" phosphopolysaccharide forming the most external layer surrounding these cells. Interactions between phage RBPs and the pellicle appear to be characterized by a moderate affinity but with long enough adhesion times (thanks to low k_{off}) to allow initiating infection. Progresses are being made to decipher the RBP/pellicle recognition mechanisms in different phages, aiming to understand the molecular determinants of this specificity.

According to the literature, a large number of phages exhibit a baseplate, beyond those infecting *L. lactis*. This probably reflects the fact that many phages use sugars as receptors. The nominal weaker affinity for polysaccharides as compared to protein/protein interactions (e.g., phage T5 pb5 binds to FhuA receptor with sub-nanomolar affinity; Flayhan et al., 2012) is compensated by a large number of receptor binding sites (18 for p2, 54 for TP901-1), although we do not know if all of them are available for binding (Desmyter et al., 2013). However, due to avidity, binding of only a few receptors should be sufficient to yield a sub-nanomolar Kd. The activation mechanism probably acts as a safety switch allowing promotion of an infection-competent metastable conformation of the virions only when the physico-chemical conditions correspond to those of the host ecosystem. The more stable rest state might be well adapted to dissemination of the virions as aerosols. This mechanism, shared by Myoviridae, may encompass a wider range of Siphoviridae beyond lactococcal phages.

ACKNOWLEDGMENTS

This work was supported, in part, by grants from the Marseille-Nice Génopole, the CNRS and the Agence Nationale de la Recherche (grants ANR-07-BLAN-0095, "Siphophages" and ANR-11-BSV8-004-01 "Lactophages").

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 24 November 2013; accepted: 04 January 2014; published online: 16 January 2014.

Citation: Spinelli S, Veesler D, Bebeacua C and Cambillau C (2014) Structures and host-adhesion mechanisms of lactococcal siphophages. Front. Microbiol. ***5****:3. doi: 10.3389/fmicb.2014.00003*

This article was submitted to Virology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 24 December 2013
doi: 10.3389/fmicb.2013.00408

The extracellular phage-host interactions involved in the bacteriophage LL-H infection of *Lactobacillus delbrueckii* ssp. *lactis* ATCC 15808

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The complete genome sequence of *Lactobacillus* bacteriophage LL-H was determined in 1996. Accordingly, LL-H has been used as a model phage for the infection of dairy *Lactobacillus*, specifically for thermophilic *Lactobacillus delbrueckii* ssp. *lactis* host strains, such as ATCC 15808. One of the major goals of phage LL-H research consisted of the characterization of the first phage-host interactions at the level of phage adsorption and phage DNA injection steps to determine effective and practical methods to minimize the risks associated with the appearance and attack of phages in the manufacture of yogurt, and Swiss or Italian hard type cheeses, which typically use thermophilic lactic acid bacteria starter cultures containing *L. delbrueckii* strains among others. This mini review article summarizes the present data concerning (i) the special features, particle structure, and components of phage LL-H and (ii) the structure and properties of lipoteichoic acids (LTAs), which are the phage LL-H receptor components of *L. delbrueckii* ssp. *lactis* host strains. Moreover, a model of the first, extracellular, phage-host interactions for the infection of *L. delbrueckii* ssp. *lactis* ATCC 15808 by phage LL-H is presented and further discussed.

Keywords: bacteriophage LL-H, lactic acid bacteria, *Lactobacillus delbrueckii*, lipoteichoic acid, phage receptor, antireceptor, phage adsorption, phage–host interaction

INTRODUCTION

The food industry benefits from the use of microbes as "work horses" in food processing, which contribute to the texture, chemical, and sensory properties of final food products. Lactic acid bacteria (LAB) are perhaps the most common bacteria applied as starter cultures for food manufacture. At an industrial scale, LAB starter-based lactic fermentations are performed in tanks with volumes of up to tens of cubic meters. The high number of starter cells comprising a single or a few strains makes these types of food processes highly susceptible to sudden appearance and attack of bacterial viruses, that is (bacterio)phages, and consequently increases the risk of the failure to control food fermentations and the quality of food products. LAB phages and phage resistance in LAB starter cultures have been intensively studied for decades because of the economic impact of the phage problems on the food industry. To obtain an in-depth scientific basis for the development of tools and approaches to minimize the risks of LAB phage infections in industrial food preparations associated with LAB fermentations, a better understanding of the origin, genetic diversity, and evolution of phages and phage biology, including phage–host interactions and phage resistance mechanisms (Samson et al., 2013), is needed.

The bacteriophage LL-H was isolated in 1972 from a whey sample originating from a problematic Emmental cheese production lot at a co-operative cheese processing plant in Hauho (Finland). The cheese starter culture employed at this dairy contained the *Lactobacillus delbrueckii* ssp. *lactis* (formerly *L. lactis*) strain LL23, which is sensitive to phage LL-H infection (Alatossava and Pyhtilä, 1980). Presently phage LL-H is one of the most thoroughly studied LAB phages and the first *Lactobacillus* phage, for which the complete genome sequence has been determined (Mikkonen, 1996; Mikkonen et al., 1996). Subsequently in the research, strain LL23 has been replaced with ATCC 15808, a more phage LL-H sensitive and widely available strain compared to LL23 strain.

STRUCTURE OF PHAGE LL-H PARTICLE

The results from electron microscopy (EM) studies on phage LL-H have revealed that this phage represents the most common morphological group among phages having an icosahedral head (capsid coat containing compactly packed linear phage DNA) and a long, non-contractile tail. A small base plate and a flexible tail fiber are located at the end of the tail, as summarized in **Figure 1** (Alatossava and Pyhtilä, 1980; Alatossava, 1987; Forsman and Alatossava, 1991; Forsman, 1994). Phage LL-H belongs to the *pac*-type phages comprising a linear ds-DNA molecule of approximately 38 kb with a 3 kb terminal repeat, packaged inside each phage capsid (Forsman and Alatossava, 1991). The linear LL-H DNA inside the capsid likely complexes with the divalent cations Ca^{2+} and/or Mg^{2+}, which are co-transported into the cell as counterions of LL-H DNA during phage DNA injection (Alatossava, 1987; Alatossava et al., 1987). Phage LL-H particles are sensitive to Tris-buffer treatment (dialysis or gel filtration with Tris-buffer), which promotes *in vitro* phage DNA ejection (Alatossava, 1982). LL-H phages readily form clusters comprising filled and empty phage (ghost) particles connected together at the ends of the tails (Alatossava and Pyhtilä, 1980).

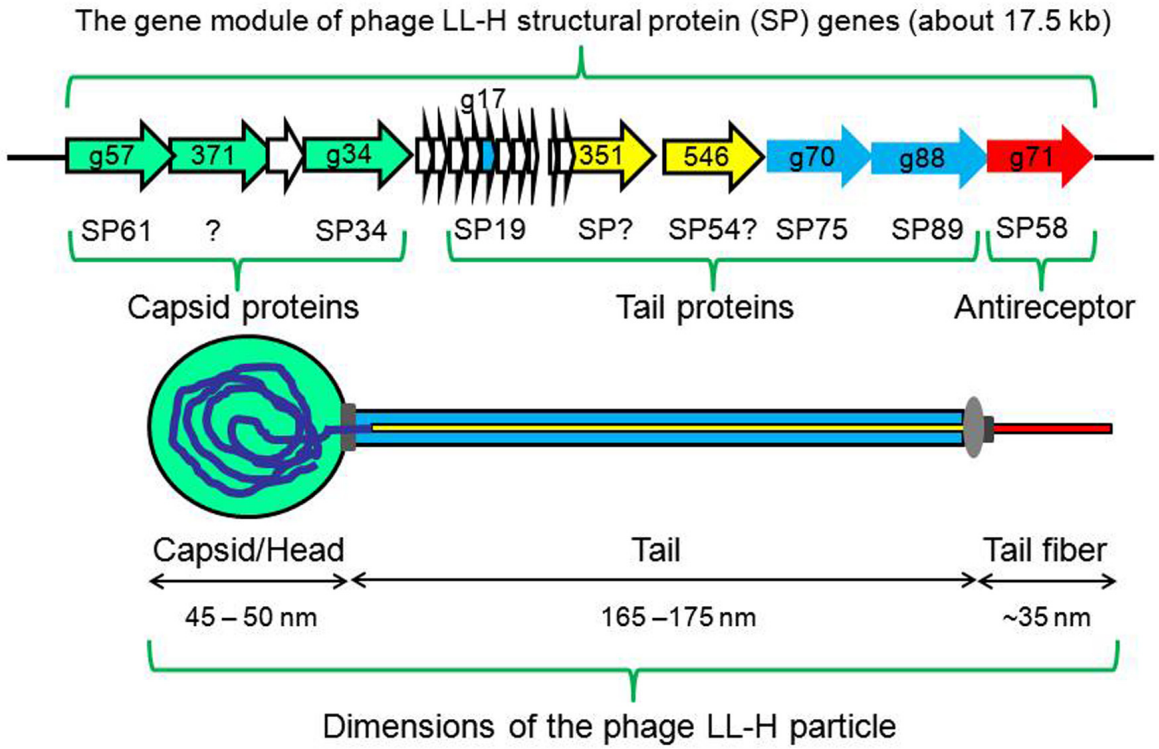

FIGURE 1 | Schematic diagram of phage LL-H particle. Genes encoding capsid and tail proteins are indicated as green and blue, respectively. Gene *g71* (red) encodes the antireceptor protein SP58. The proteins coded by genes *orf351* and *orf546* (yellow) are considered as tape measure protein (TMP) homologs, likely localized to the core of the tail. The base plate is indicated as gray.

Based on the SDS-PAGE analysis of twice-purified (by CsCl density gradient centrifugation) LL-H phage particles, nine different structural proteins of 89, 75, 61, 58, 54, 34, 23, 19, and 14 kDa have been identified (Alatossava, 1987; Forsman and Alatossava, 1991). Among these, six phage structural proteins have been further characterized at the gene level (**Figure 1**): gene *g34* encodes the major capsid protein SP34; gene *g57* encodes the minor capsid protein SP61; gene *g17* encodes the major tail protein SP19; genes *g70* and *g88* encode the two minor tail proteins, SP75 and SP89, respectively; and gene *g71* encodes the tail fiber antireceptor protein SP58 (Gp71) (Trautwetter et al., 1986; Vasala et al., 1993; Mikkonen and Alatossava, 1994; Vasala, 1998; Ravin et al., 2002). No phage genes have been identified for the 54, 23, and 14 kDa LL-H structural proteins. These proteins may represent the proteolytic products of some other phage structural proteins or may be of bacterial origin. We re-sequenced the region between *orf351* and *orf360* described previously (Mikkonen and Alatossava, 1994; Mikkonen, 1996), and observed an additional G (GGG instead of GG) after the nt position 13888 (Mikkonen, 1996); consequently a single larger gene, *orf546,* was identified (**Figure 1**). Gene *orf546* could potentially encode the observed structural protein SP54 that is present in the phage LL-H particle but is absent in the LL-H ghost particle (Trautwetter et al., 1986). The N-terminal domain of phage LL-H ORF351 (aa-residues 20–105) shows amino acid sequence homology with the domains of several phage tape measure proteins (TMP) including *L. delbrueckii* phage c5 putative TMP (Riipinen, 2011; Riipinen et al., 2011). In addition, the C-terminal end of phage LL-H ORF546 containing peptidoglycan (PG)-hydrolysing domain features, shows aa sequence homology with a putative TMP of *L. delbrueckii* phage JCL1032 (Riipinen, 2011; Riipinen et al., 2011). Accordingly, both ORF351 and ORF546 proteins have been localized to the core of the phage LL-H tail, similar to TMPs (Plisson et al., 2007). However, instead of a single multifunctional, large TMP (Boulanger et al., 2008), the TMP-associated PG-hydrolysing and cytoplasmic membrane (CM)-binding activities preceding phage DNA transfer are mediated through separate TMP homologs, proteins ORF546 (SP54?) and ORF351, respectively, in phage LL-H (**Figure 1**).

STRUCTURE AND PHAGE RECEPTOR PROPERTIES OF *Lactobacillus delbrueckii* LIPOTEICHOIC ACIDS

The first step of the phage infection cycle is the phage adsorption, the specificity of which is determined by the host bacterial surface component(s) and the phage component(s), receptor(s), and antireceptor(s), respectively. Furthermore, phage adsorption can be divided in two phases: a reversible phase and an irreversible phase. Among Gram-positive eubacteria, peptidoglycans, wall teichoic acids (WTA), lipoteichoic acids (LTA), and CM-associated proteins have been reported as phage receptor molecules. Proper genetic changes like spontaneous mutations affecting the structures of these molecules can prevent the adsorption of a particular phage and consequently increase the phage resistance of the

mutated bacterial strain. However, the coevolution of phages with host bacteria facilitates mutations. For example, mutations in the antireceptor gene could change the host ranges of these mutant phages, and could potentially make the receptor mutant strains sensitive to the infections with these types of antireceptor phage mutants.

Both the phage receptor mutants of strain ATCC 15808 (the strain Ads-5) and the antireceptor mutants of phage LL-H (the strain LL-H-a21) have been isolated and further characterized (Ravin et al., 2002; Räisänen et al., 2004, 2007). The phage receptor mutant strain Ads-5 does not adsorb wild-type phage LL-H, but rather facilitates the effective adsorption of the host range phage mutants such as LL-H-a21. Each of the five host range phage mutants of LL-H studied contains a single nucleotide change at the 3′-end of gene *g71*, which encodes a mutant protein with a single aa substitution (Asn to Lys, Ala to Ser, or Gln to His) in the C-terminal end [in the region 380–543 aa of the 656 aa-protein SP58 (Gp71)]. Consequently, *g71* has been designated as the antireceptor-encoding gene. The structural analyses of LTAs from ATCC 15808 and Ads-5, the phage receptor mutant strain of ATCC 15808, have revealed a significant difference: the polyglycerolphosphate backbone of the LTA from Ads-5 lacks the single glucose moiety located most probably at the surface end of the LTA from ATCC 15808. Other structural features of LTAs, such as the levels of D-alanylation, average numbers of glycerolphosphate repeats, and the ratios and compositions of fatty acids linked to the triglucose moiety of the glycolipid anchor were not changed (Räisänen et al., 2007). The results of phage inactivation studies using purified LTA preparations suggest that the surface glucose substituted LTA is required for the specific reversible adsorption and enough free, nonsubstituted glycerol residues (allowing local negative charge in the LTA backbone) for the irreversible adsorption of wild-type LL-H. For the host range phage LL-H mutants, both the surface glucose-substituted and surface glucose-free forms of LTAs are equally functional as phage receptors (Räisänen, 2007). Thus, the extension of the host range may occur at the expense of the specificity of the phage receptor.

A MODEL OF PHAGE LL-H ANTIRECEPTOR – HOST LTA INTERACTIONS

The structural properties of the phage LL-H particle and the genetic, biochemical, and electron microscopic data on the phage LL-H antireceptor/fiber, the ATCC 15808 LTA as phage receptor, and the phage LL-H infectivity and stability properties suggest, for

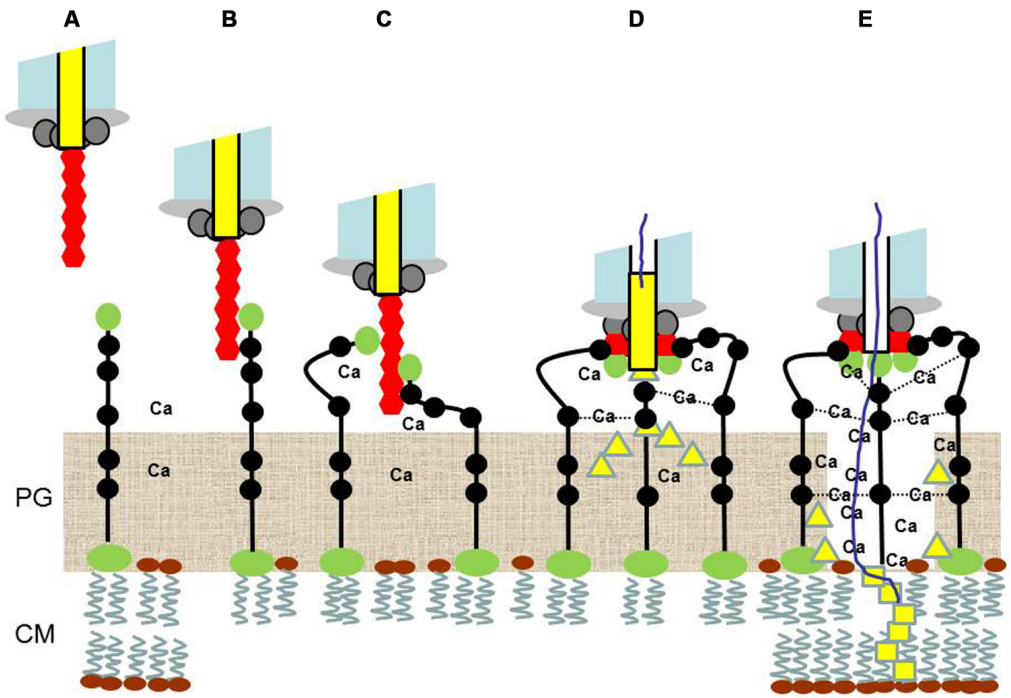

FIGURE 2 | A model of phage LL-H antireceptor-*Lactobacillus delbrueckii* ssp. *lactis* ATCC 15808 lipoteichoic acid (LTA) interactions during the extracellular phase of phage infection [steps (A–E)]. **(A)** The LL-H tail fiber (red) is suggested to comprise six antireceptor protein (SP58) subunits. **(B,C)** Each antireceptor subunit initially interacts (reversible adsorption) with the top glucose moiety (green), followed by one of the negatively charged top phosphate groups (black) of LTA (irreversible adsorption) to facilitate the tail fiber attachment to the ends of up to six LTA molecules outside the peptidoglycan (PG) layer. **(D)** SP58 subunits-LTA interactions promote the tail fiber rearrangement into a ring structure attached to the end of the tail base for the release of the TMP homologs ORF546 (SP54?) and ORF351 from the tail core into the space restricted by the SP58-bound LTA molecules and further stabilized with calcium (Ca) bridges. **(E)** The PG-degrading activity of ORF546 proteins (yellow triangle) produces additional space for the formation of the stable Ca-LTA channel between the end of the tail base and the cytoplasmic membrane (CM). The Ca-LTA channel provides a gateway for ORF351 proteins (yellow square) to interact with the CM, and to further guide the transfer of the linear phage LL-H DNA complexed with calcium, through the CM into the cytoplasm of the infected host bacterial cell.

the extracellular interactions between phage LL-H antireceptor and LTAs of the ATCC1 5808 host strain, a model described in **Figure 2**. The tail fiber, which is approximately 35 nm in length (Forsman and Alatossava, 1991; Forsman, 1994), is considered as a flexible hexapolymer of the antireceptor protein SP58 encoded by gene *g71* of phage LL-H. The C-terminal end of each antireceptor protein subunit contains one domain responsible for the reversible, specificity-determining binding to the surface end of the LTA, primarily mediated through hydrogen bonds to the glucose moiety. The second domain of the antireceptor protein SP58 ensures the irreversible binding to the negatively charged glycerol phosphate group(s) (no or low local D-Alanylation of glycerol phosphate repeats) close to the surface end of the LTA, possibly mediated through ionic bonds. Altogether, up to six LTA molecules can bind to the single phage tail fiber, promoting the rearrangement of the fiber to form a ring structure with a maximum diameter of 11 nm (i.e., 35 nm/π) attached to the tail base by the interactions of the six antireceptor protein subunits. The occurrence of the phage tail end-LTAs complexes could explain the observed heterogeneous structures and clusters of phage tail ends of negatively stained LL-H phage and empty LL-H ghost particles (e.g., Alatossava and Pyhtilä, 1980). The ring arrangement of the six antireceptor subunits suggests that the TMP homologs ORF546 (SP54?) and ORF351 are released from the core of the tail before the release of linear phage DNA. The muranolytic activity of ORF546 degrades PG inside the space of the LTA molecules (up to six) attached to the hexameric antireceptor (SP58) ring, facilitating the free movement of these attached LTA molecules and the subsequent formation of a stable calcium-LTA channel between the tail base end and CM as a tail extension. Phage LL-H infectivity is highly dependent on the external calcium or magnesium concentration, with an optimum of nearly the Ca/Mg solubility limits of the medium, 20–40 mM, which is approximately one log-unit higher than the Ca/Mg optimum required for phage LL-H adsorption (Alatossava, 1987). Accordingly, the requirements for Ca-LTA channel formation could reflect the observed high external calcium (or alternatively magnesium) concentration for optimal phage LL-H infectivity. Moreover, the proposed Ca-LTA channel would act as a gateway to the CM for ORF351, which contains two putative transmembrane motifs (residue regions 140–170 and 250–270) following the N-terminal tape measure domain. These interactions are likely required for the successful and effective transfer of linear phage DNA as a Ca-complex through the CM into the cytoplasm. The observed m.o.i. (multiplicity of infection)-dependent influx of calcium into the infected cell during the first minutes of phage infection (Alatossava et al., 1987) supports this model.

PERSPECTIVE

A model of the extracellular interactions between the phage antireceptor and LTA receptors of the host has been proposed. The suggested formation of a stable calcium-LTA channel as the connecting structure between the phage LL-H tail base and the host CM could reflect the observed high calcium dependency for optimal LL-H infectivity. The Ca-LTA channel formation could exist not only in the case of phage LL-H, but also more generally among Gram-positive Ca-dependent phages, which do not contain phage lysin as an external structural tail base component.

ACKNOWLEDGMENTS

This work was supported through a grant (received on September 25, 2012) from the Finnish Cultural Foundation to Tapani Alatossava.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 October 2013; accepted: 10 December 2013; published online: 24 December 2013.

Citation: Munsch-Alatossava P and Alatossava T (2013) The extracellular phage-host interactions involved in the bacteriophage LL-H infection of Lactobacillus delbrueckii ssp. lactis ATCC 15808. Front. Microbiol. ***4****:408. doi: 10.3389/fmicb.2013.00408*

This article was submitted to Virology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 10 April 2014
doi: 10.3389/fmicb.2014.00159

Phages of *Listeria* offer novel tools for diagnostics and biocontrol

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Historically, bacteriophages infecting their hosts have perhaps been best known and even notorious for being a nuisance in dairy-fermentation processes. However, with the rapid progress in molecular microbiology and microbial ecology, a new dawn has risen for phages. This review will provide an overview on possible uses and applications of *Listeria* phages, including phage-typing, reporter phage for bacterial diagnostics, and use of phage as biocontrol agents for food safety. The use of phage-encoded enzymes such as endolysins for the detection and as antimicrobial agent will also be addressed. Desirable properties of candidate phages for biocontrol will be discussed. While emphasizing the enormous future potential for applications, we will also consider some of the intrinsic limitations dictated by both phage and bacterial ecology.

Keywords: bacteriophage, tools, diagnostics, typing, biocontrol, endolysins

INTRODUCTION

Bacteriophages affect the world in numerous ways. They represent parasites of bacteria, without own metabolism, relying on the host cell to provide energy and resources for their own replication. In terms of quantity, phages are the most abundant self-replicating entities on our planet, and outnumber their hosts by a factor of at least 10:1. This results in an estimated population of 10^{30}–10^{31} phage particles existing on the planet at any given time (Wommack and Colwell, 2000). In order to maintain this number, an estimated 10^{25} infections happen every second (Pedulla et al., 2003). In any environment where bacteria play an important role it therefore stands to reason that bacteriophage will have an impact on that environment. In the worlds' oceans and seas cyanobacteria play a vital role in primary production and most work focusing on ecological impact of phages has focused on their role in nutrient cycles. However, it should be obvious that, next to protozoa, phages are the only natural predators of bacteria and thus play an essential role in controlling bacterial populations everywhere. Bacteriophages also influence human lives in various other ways. Wherever bacteria are used in fermentation processes, be it for food or production of other compounds, phages can affect the end-result by infecting and lysing the desired bacteria. In the biological sciences, bacteriophages have been instrumental in building our understanding of fundamental genetic principles, such as the concept of the operon, functioning of promoters and the restriction-modification, one of the most valuable and versatile tools in molecular biology. Phages can contribute to the virulence of bacterial pathogens in two ways. General transduction is a process where instead of phage-DNA host-DNA is packaged into infectious phage-like particles and this host DNA is subsequently injected into a susceptible host where it may integrate into the chromosome through homologous recombination. Whenever this transduced DNA contains virulence genes the infected bacteria can have altered virulence. Although other strategies for survival and replication exist, most phages can be separated into temperate or virulent phages. The latter are often termed lytic or strictly lytic as well. This lifestyle involves obligate production of new phages after infection resulting in lysis of the host and release of progeny phage. Temperate phages can do this but alternatively they can integrate their genetic information into the host chromosome. Lysogenic conversion is the phenomenon where temperate phages alter the virulence of the infected host after DNA integration. Examples of bacteria that rely on phage (genes) for their full pathogenic potential are *Vibrio cholera*, which is only pathogenic when it the cholera toxin is introduced by phage CTXϕ, the diphtheria toxin of *Corynebacterium diphteriae* is also encoded by a phage, and shiga-toxin production in Enterobactericeae originates from genes on temperate phages integrated into the genomes of these bacteria (Freeman, 1951; Smith et al., 1983; Waldor and Mekalanos, 1996). Many more examples can be found in the literature. Lastly, phages have long been used in medical applications. Phage-typing has long been employed to conduct epidemiological studies of bacterial infection.

Over the past two decades, there has been a huge revival of interest in studying phages, but for other reasons. The emergence and increase in antibiotic-resistant bacteria and the possibility of replacing chemical drugs with phage or phage-encoded enzymes has been one of the main driving forces behind this renaissance. The fundamental insights which the genomics era has provided us with also led to the development of various tools based on phages or their components. Phage protein based detection of bacteria is now commercially available in the form of Vidas UP® technology, which employs recombinant host recognition proteins for *in vitro* diagnostics (IVD) of *Escherichia coli* and *Salmonella*. Phages have also been considered and applied since many decades to treat bacterial infections. This practice was and still is most common in the former Soviet Union, and to this day, phage preparations to treat a variety of infections are still produced in

the Republic of Georgia. In the Western world, phage products for biocontrol are currently being commercialized for agricultural applications and food applications. Phage-derived proteins have also been commercialized and a number of commercial companies are investigating phage or phage-derived products for use in medical applications. The following chapters will focus on the impact of phage studies on our understanding of the genus *Listeria* with emphasis on *Listeria monocytogenes* as well as phage-derived tools for the detection and biocontrol of this important foodborne pathogen.

THE GENUS *Listeria*

Listeria spp. are small Gram-positive, flagellated rods with a ubiquitous distribution in the environment. Their principle lifestyle is saprophytic and thus the bacteria are frequently associated with plant material. Nine species are currently recognized, and they are *L. monocytogens*, *L. ivanovii, L. innocua, L. marthii, L. rocourtiae, L. seeligeri, L. welshimeri L. fleischmannii,* and *L. grayii.* Most research has been undertaken with respect to the human pathogen *L. monocytogenes* (Glaser et al., 2001; Graves et al., 2010; Leclercq et al., 2010; Bertsch et al., 2013). In healthy individuals, the infection (termed listeriosis) often manifests itself as a gastroenteritis, but in the very young, old, pregnant, and immunocompromised (a risk group commonly referred to as YOPIs), the bacteria can penetrate the intestinal tract and cause systemic infections including the central nervous system, and stillbirth or abortion in pregnant women (Vázquez-Boland et al., 2001). The disease is caused by the consumption of food contaminated with *L. monocytogenes.* The bacterium is a psychrotroph and can grow at refrigeration temperatures, and tolerates a wide range of pH and salt concentrations, rendering even low contaminations at production level dangerous if the shelf life of the food is longer than a few days (Gandhi and Chikindas, 2007). Many countries have strict regulations on the number of *L. monocytogenes* cells that are allowed in a given food, and zero-tolerance policies are in place in the US and Oceania. Phage therapy of Listeriosis is of course no option, as the bacteria multiply as intracellular pathogens, and cannot be reached by phages. Even antibiotics to which the bacteria are usually highly sensitive need to be administered at relatively high doses over prolonged periods of time in order to establish sufficient intracellular concentration. However, phage can be used both for the detection of this pathogen, and in controlling their presence in food processing equipment and on ready-to-eat products.

BACTERIOPHAGES OF *Listeria*

Listeria phages have been isolated from several of sources, including sewage plants, silage, food processing environments, and from lysogenic strains (Loessner and Busse, 1990; Hodgson, 2000; Kim et al., 2008; Arachchi et al., 2013). In total, some 500 phages specifically infecting *Listeria* have been identified, while only a limited number has been fully characterized on the molecular and genomic level (Loessner et al., 2000; Zimmer et al., 2003; Carlton et al., 2005; Dorscht et al., 2009; Schmuki et al., 2012). Phages have been reported to be able to infect other species. These studies revealed extensive mosaicism between many *Listeria* phages. All known *Listeria* phages belong to the order *Caudovirales*, which are tailed viruses with double stranded DNA. Podoviruses feature very short tails, while myoviruses have long, non-flexible contractile tail and siphoviruses have long, flexible tails. While members of both the *Siphoviridae* and *Myoviridae* are common, no *Podoviridae* infecting *Listeria* have yet been found and reported. The majority of the described phage are temperate, being able to integrate into the host genome. In fact, many *Listeria* strains are lysogenic, sometimes carrying multiple prophages, while most also harbor defective prophages. Many *Listeria* strains produce and release so-called monocins, which represent defective prophages, often recognized as lytic particles resembling phage tail structures (Zink et al., 1994, 1995). As in other bacteria, exposure to DNA-damaging agents such as UV light or Mitomycin C can induce the lytic cycle and lead to production of infective phage (Loessner, 1991). *Listeria* temperate phages generally display narrow host ranges, infecting only a small percentage of strains. This is in part caused by homoimmunity, i.e., resistance to a particular phage based on presence of a compatible repressor protein encoded by the same or a related phage within the bacterial cell. It is also noteworthy that the temperate phages of *Listeria* appear to be largely serovar-specific. It has been shown that this is based upon cell wall ligands, which the temperate phages recognize and attach to infect their hosts. They are serovar-specific sugar substituents of the polyribitol-phosphate teichoic acids on the *Listeria* cell wall (Wendlinger et al., 1996). In contrast, the large virulent phages such as A511 have been shown to use the cell-wall peptidoglycan backbone itself as a primary receptor (Wendlinger et al., 1996; Habann et al., 2014). Phages specifically infecting the different major serovar groups (1/2, 4, 5, and 6) have been identified, while no phages for *L. monocytogenes* serovar 3 or *L. grayii* strains have yet been found. Very little data exist on phenotype conversion or alteration following lysogenization. A recent study shows that prophage integration into the *Listeria comK* gene hinders cells escaping from macrophage phagosomal environments. In this case, the prophage may be excised during intracellular growth without generating progeny phage, integrity of *comK* is reconstituted, and following a cascade of events triggered by the ComK protein, the bacteria can eventually escape (Rabinovich et al., 2012). Another study shows that cells which harbor a phage integrated into *comK* display enhanced persistence and higher cell densities under meat processing plant conditions compared to strains lacking the phages. However, no explanation is offered and the underlying mechanisms causing this phenomenon remain obscure (Verghese et al., 2011). The less frequent virulent phages of *Listeria* fall into three groups. The large (∼140 kb) Myoviruses P100 and A511 (Carlton et al., 2005; Klumpp et al., 2008) feature exceptionally broad host ranges, while the smaller siphoviruses P35 and P40 (around 40 kb) and the recently described, slightly larger (70 kb) phage designated P70 (Dorscht et al., 2009; Schmuki et al., 2012) still infect most members of given serovar groups. Virulent phages, i.e., those that will always kill their host cells are of course most useful when phage-based biocontrol is the aim. In contrast, generalized transducing phages, even though they may feature a virulent lifestyle such as P35 (Hodgson, 2000), are not desirable for application in biocontrol because they may tranfer genetic material, including virulence factors from one strain to another (Hagens and Loessner, 2010). Preferred are members of

a specific group of the large, virulent myoviruses closely related to P100 and A511, which have been isolated from sources in Europe, the US and New Zealand (Zink and Loessner, 1992; Carlton et al., 2005; Kim et al., 2008; Bigot et al., 2011; Arachchi et al., 2013).

The natural property of phage to specifically target and kill bacterial cells of a certain species or genus has been fairly well exploited with respect to *Listeria*. As mentioned above, phage therapy of *L. monocytogenes* infections is not possible. However, prevention of the disease by anti-*Listeria* control measures during and after food processing is highly effective and desirable. Phages used for biocontrol purposes should be virulent and feature a broad host range, i.e., infect and kill as many target strains as possible. They must also be unable to transduce, and should ideally be propagated on a non-pathogenic production strain, or alternatively needs to be purified from the bacterial lysate. As of now, two *Listeria* phage products are commercially available: a broad host range myovirus is marketed as "Listex^{TM} P100," and was granted GRAS (Generally Recognized As Safe) status by FDA and USDA in 2007 for use in all food products. Several studies have demonstrated its efficacy in foods such as cheese, RTE (ready to eat) meats and poultry, fruits, vegetables, and smoked fish, either alone or in combination with a growth limiting antimicrobial (Carlton et al., 2005; Soni and Nannapaneni, 2010a; Soni et al., 2010, 2012; Chibeu et al., 2013; Oliveira et al., 2014). Another study showed its efficacy on stainless steel surfaces, achieving more than five logs bacterial kill (Soni and Nannapaneni, 2010b). Another study used phage A511, closely related to P100, and confirmed its effectiveness in various RTE foods and surface ripenend soft cheeses (Guenther et al., 2009; Guenther and Loessner, 2011). Another product is termed "ListShield," and is also FDA and USDA approved. It consist of a cocktail of several phages, and its efficacy was investigated in two studies using fresh-cut produce in combination with the bacteriocin Nisin and as a spray application on honey-dew melons (Leverentz et al., 2003, 2004). An alternative approach to suspended phage is directed immobilization of the viral particles on cellulose membranes, which has been proposed as a possible intervention strategy against *Listeria* on packaging material (Anany et al., 2011).

TOOLS BASED ON *Listeria* PHAGE

Table 1 lists several *Listeria* phage-derived applications. The first widely used application was in phage typing. This is an inexpensive, low-tech and relatively quick tool to differentiate strains in epidemiological and microbial source-tracking studies. While being increasingly replaced by modern and more sophisticated molecular methods, it is yet not likely to become completely obsolete because of its simplicity and low cost. Phage typing had a prominent role in demonstrating food to be the primary cause of listeriosis in humans (Fleming et al., 1985). A number of different phage sets were developed (Audurier et al., 1979, 1984; Rocourt et al., 1985; McLauchlin et al., 1986; Loessner and Busse, 1990; Loessner, 1991; Estela and Sofos, 1993; Gerner-Smidt et al., 1993; van der Mee-Marquet et al., 1997). However, since *Listeria* serovar 3 strains appear resistant to phage infection, not all isolates can be typed. However, almost all strains that occur in foodborne outbreaks feature either serovar 1/2 or 4b, and are mostly susceptible to phage infection and can therefore generally be differentiated using phage typing (van der Mee-Marquet et al., 1997). Transducing phages, while not suited for biocontrol purposes, have been used to transfer genetic traits from one strain to another, allowing genetic manipulation and phenotype investigations (Freitag, 2000; Hodgson, 2000). Lauer and coworkers provided specific *E. coli*/*Listeria* shuttle vectors designed to integrate into the *Listeria* chromosome after transformation. Here, site-specific integration by *Listeria* phage integrase has been employed to mediate single-copy, selection-free integration of genes placed on the vectors. As a proof of concept, the approach allowed successful complementation of *actA* and *hly* knockouts (Lauer et al., 2002). Derivatives of the initial pPL2 vector have

Table 1 | *Listeria* phage-derived tools.

Phage or phage components	Application	Reference
Large sets of different phages (broad and narrow host range)	Phage typing	Audurier et al. (1979, 1984), Rocourt et al. (1985), McLauchlin et al. (1986), Loessner and Busse (1990), Loessner (1991), Estela and Sofos (1993), Gerner-Smidt et al. (1993), van der Mee-Marquet et al. (1997)
Single phages or cocktails (broad host range)	Biocontrol (targeted killing in foods)	Leverentz et al. (2003, 2004), Carlton et al. (2005), Guenther and Loessner (2011), Guenther et al. (2009), Soni et al. (2010, 2012), Soni and Nannapaneni (2010a), Chibeu et al. (2013),Oliveira et al. (2014)
Reporter phages (broad host range)	Diagnostics (detection)	Loessner et al. (1996, 1997), Hagens et al. (2011)
Phage endolysins and functional domains	Diagnostics	Kretzer et al. (2007), Schmelcher et al. (2010), Walcher et al. (2010), Tolba et al. (2012)
Phage tail fibers	Immobilization and detection	Junillon et al. (2012), Habann et al. (2014)
Endolysins and modified endolysins	Antimicrobial	Fischetti (2010), Schmelcher et al. (2012)
Phage integrase genes	Site-specific integration vectors	Lauer et al. (2002)

since been used in many studies addressing *L. monocytogenes* phenotypes and virulence properties, have been successfully employed to monitor promoter activity in *in vivo* infection studies (Lenz and Portnoy, 2002; Lenz et al., 2003; Grundling et al., 2004; Bron et al., 2006), and were fundamental in elucidating the mechanisms of L-form formation in *L. monocytogenes* (Dell'Era et al., 2009).

Rapid and efficient detection of a *Listeria* contamination is critical with respect to food safety, and for compliance of food production with regulations concerning the presence of *L. monocytogenes* in foods and on food-contact surfaces. The high specificity of phages combined with the fact that viruses only multiply in viable and active host cells has sparked the development of phage amplification-based assays for the detection of various pathogens, including *Listeria*. One possible use is measuring an increase of *Listeria* phages after incubation with a contaminated sample (Bakulov et al., 1984). However, genetically modified reporter phages carrying a gene encoding an easy-to-measure product or activity offer more interesting and versatile alternatives. The reporter gene product will only be made following successful phage infection and gene expression. *Listeria* reporter phage A511::*luxAB* transduces a *luxAB* gene fusion coding for bacterial luciferase (Loessner et al., 1996), and its efficacy in rapid detection of low-level *Listeria* contaminations from various foods has been demonstrated (Loessner et al., 1997). The same phage was used to construct A511::*celB*, featuring a thermophilic glycosidase of *Pyrococcus furiosus*. Reporter genes from thermophiles feature an exceptional ease-of-use, and allow a wide range of substrates and assay formats to choose from (Hagens et al., 2011).

Rather than employing complete phages as antimicrobials and for diagnostics, proteins and peptides from phage may also be used for this purpose. Phage-encoded cell-wall-hydrolyzing enzymes are particularly useful. In order to digest the bacterial cell wall prior to release of phage progeny, most phages produce a class of enzymes termed endolysins. These enzymes feature a two-domain structure with an N-terminal catalytic domain and a C-terminal cell wall-binding domain (CBD) that binds with high affinity to cell wall-associated ligands, in order to direct the catalytic activity to its target site, and at the same time prevent collateral damage and lysis of yet uninfected potential host cells (Loessner et al., 1995). Such endolysin peptidoglycan hydrolases have a huge potential as antimicrobial reagents against Gram-positive pathogens, which has been reviewed elsewhere (Fischetti, 2010; Schmelcher et al., 2012). Yet, application of *Listeria* phage endolysins as highly specific antimicrobial agent in foods is hampered by the specific requirements of these enzymes, in terms of pH and salt concentration. Endolysins from *Listeria* phages can, however, be useful for removing *Listeria* occurring on food-contact surfaces and in biofilms.

Due to the high binding affinity and specificity of the endolysin CBD, these protein domains have been explored and used as versatile tools for diagnostics and identification of *Listeria*. Studies have shown that CBDs immobilized on paramagnetic beads are very effective in order to separate target cells from dilute suspensions, and the technique can be elegantly combined with different secondary diagnostic steps (Kretzer et al., 2007; Walcher et al., 2010). A recent study investigated detection of *Listeria* cells using electrochemical impedance spectroscopy (EIS), which can measure bacteria captured by the CBD molecules immobilized on a gold screen printed electrode (SPE; Tolba et al., 2012). Differently colored fluorescent proteins fused to CBDs with different recognition and binding spectra allow for rapid and multiplexed detection and differentiation of *Listeria* strains by fluorescence microscopy (Schmelcher et al., 2010). As an alternative approach, *Listeria* phage tail fiber proteins coupled to a solid support can also be used to bind target cells for primary enrichment and subsequent detection (Junillon et al., 2012).

In summary, the past years have demonstrated that phages infecting *Listeria* and other bacteria (Hagens and Loessner, 2010) provide us with numerous novel tools for various interesting approaches. The evolution of molecular microbiology and genetic engineering as technology platform, together with insights into phage biology, have been combined in interesting ways, and it is likely that this process will continue and more tools harnessing the properties and incredible biological specificity of bacterial viruses will be developed and available in the future.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 05 February 2014; accepted: 25 March 2014; published online: 10 April 2014. Citation: Hagens S and Loessner MJ (2014) Phages of Listeria offer novel tools for diagnostics and biocontrol. Front. Microbiol. ***5****:159. doi: 10.3389/fmicb.2014.00159*
This article was submitted to Virology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 28 April 2014
doi: 10.3389/fmicb.2014.00184

Clostridium difficile phages: still difficult?

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Phages that infect *Clostridium difficile* were first isolated for typing purposes in the 1980s, but their use was short lived. However, the rise of *C. difficile* epidemics over the last decade has triggered a resurgence of interest in using phages to combat this pathogen. Phage therapy is an attractive treatment option for *C. difficile* infection, however, developing suitable phages is challenging. In this review we summarize the difficulties faced by researchers in this field, and we discuss the solutions and strategies used for the development of *C. difficile* phages for use as novel therapeutics. Epidemiological data has highlighted the diversity and distribution of *C. difficile*, and shown that novel strains continue to emerge in clinical settings. In parallel with epidemiological studies, advances in molecular biology have bolstered our understanding of *C. difficile* biology, and our knowledge of phage–host interactions in other bacterial species. These three fields of biology have therefore paved the way for future work on *C. difficile* phages to progress and develop. Benefits of using *C. difficile* phages as therapeutic agents include the fact that they have highly specific interactions with their bacterial hosts. Studies also show that they can reduce bacterial numbers in both *in vitro* and *in vivo* systems. Genetic analysis has revealed the genomic diversity among these phages and provided an insight into their taxonomy and evolution. No strictly virulent *C. difficile* phages have been reported and this contributes to the difficulties with their therapeutic exploitation. Although treatment approaches using the phage-encoded endolysin protein have been explored, the benefits of using "whole-phages" are such that they remain a major research focus. Whilst we don't envisage working with *C. difficile* phages will be problem-free, sufficient study should inform future strategies to facilitate their development to combat this problematic pathogen.

Keywords: *Clostridium difficile*, phage therapy, phage evolution, genomics, lysogeny, gut pathogen, nosocomial

C. difficile PATHOGENICITY, RIBOTYPES, AND EPIDEMIOLOGY

Over the last few decades the enteric bacterium *Clostridium difficile* has emerged as an important nosocomial pathogen in clinical settings globally, and in particular in Europe, the USA, Canada, and Australia (Kuijper et al., 2006). Despite a general trend in falling case numbers in these countries, *C. difficile* infection (CDI) remains a serious problem. For example there are an estimated 250,000 cases of CDI annually in the USA which result in approximately 14,000 deaths [Centers for Disease Control and Prevention (CDC), 2013]. In addition to the human cost of the disease, the financial costs of treating and managing the infection are significant, with an estimated annual cost of $800 million in the USA and €3000 million in Europe (Bouza, 2012). Number of CDI cases in the UK decreased from 55,498 in 2007 to 14,687 in 2013 (Public Health England, 2013), and this reduction is thought to be attributed to the enormous effort that has been put into CDI (*C. difficile* infection) management strategies such as modified infection control procedures, antibiotic stewardship, and mandatory reporting (Hughes et al., 2013). Therefore it is of concern that despite these efforts, CDI remains a major healthcare challenge.

Clostridium difficile infection is generally associated with the production of up to three toxins; toxin A and toxin B, which are encoded on a pathogenicity locus; the PaLoc, and the *C. difficile* binary toxin (CDT; Rupnik et al., 2009). These toxins disrupt the epithelial cell layer of the colon and the resulting inflammatory response contributes to the disease pathology. Symptoms range from mild to serious diarrhea and, less commonly, to the development of pseudomembranous colitis and toxic megacolon which can be fatal (Libby and Bearman, 2009).

Several CDI epidemics have been linked to specific ribotypes such as R027 and R078 (McDonald et al., 2005; Goorhuis et al., 2008), but 100s of different ribotypes have been identified (Wilcox et al., 2012). Ribotyping is a method of assigning strain type based on the amplification of the intergenic region between the 16S and 23S rRNA gene, of which *C. difficile* has multiple copies (O'Neill et al., 1996). The use of next generation sequencing (NGS) technology has revealed the genomic diversity of important ribotypes, such as R027 (Stabler et al., 2009),

Abbreviations: CDI, *Clostridium difficile* infection; CDS, coding DNA sequence; CDT, *Clostridium difficile* binary toxin encoded by two genes *cdtA* and *cdtB*; CRISPR/Cas, a form of RNA based adaptive bacterial immunity to foreign DNA, including phages and plasmids; HGT, horizontal gene transfer; MM, LTM, SMV and SV, abbreviations used to describe the main four morphological types of *C. difficile* phage observed; NGS, next generation sequencing; PaLoc, pathogenecity locus of *C. difficile* containing genes involved in toxicity; *tcdA, tcdB, tcdE, tcdC* and *tcdR*; R027, epidemic ribotype (strain) of *C. difficile*; RCDI, recurrent *C. difficile* infection; SNP, single nucleotide polymorphism; TEM, transmission electron microscopy; TcdA and TcdB, two toxins of *C. difficile* encoded on the PaLoc.

and one study has mapped the evolution and spread of this ribotype in epidemics across the world highlighting their acquisition of mobile genetic elements and antibiotic resistance genes (He et al., 2012).

The ability of *C. difficile* to form endospores permits its transmission and persistence within clinical settings (Vonberg et al., 2008). In contrast to nosocomial cases, a proportion of patients with CDI acquire *C. difficile* from sources outside the hospital environment (Eyre et al., 2012). The bacterium can colonize individuals asymptomatically, and has reservoirs associated with livestock, food and the natural environment (e.g., Hall and O'Toole, 1935; al Saif and Brazier, 1996; Metcalf et al., 2010, 2011; Zidaric et al., 2010; Pasquale et al., 2011; Hargreaves et al., 2013b). CDI has been suggested to be a zoonotic disease (Rupnik, 2007), and the cross-over of ribotypes between sources has been observed (Janezic et al., 2012) as well as strain transmission between livestock and humans (He et al., 2012). Establishing the source of disease and ecology of this pathogenic species is important for understanding *C. difficile*'s emergence, predominance, and pathology in clinical settings.

NOVEL WAYS TO COMBAT *C. difficile* INFECTIONS

Current treatment of CDI is with one of three antibiotics: vancomycin, metronidazole, or fidaxomicin; however, treatment failure and recurrent *C. difficile* infection (RCDI) can occur after treatment with any of these antibiotics (Surawicz et al., 2013). The consequences of CDI and its continued clinical prevalence, combined with limited treatment options, have motivated research into alternative therapies to treat infections caused by this bacterium. These include new antibiotics, antimicrobial peptides, bacteriocins, molecular inhibitors such as quorum sensing and riboswitch ligands, toxin targeting molecules such as antibodies, and the use of other bacteria as probiotics or fecal transplants. These approaches are at various stages of development and are discussed in a recent review and are not considered further here [see review by (Zucca et al., 2013)].

Our review focuses on bacteriophage (phage) therapy to treat *C. difficile*. The use of phages as antimicrobials to treat a range of bacterial diseases was developed shortly after their discovery in 1917, and their use as established therapeutics in some countries is well documented (Kutter and Sulakvelidze, 2004). Many reviews have been written on the efficacy and safety of phage therapy (e.g., Abedon et al., 2011; Meaden and Koskella, 2013), and phages for several infectious diseases have had notable successes and are at the stage of clinical trial testing (Brüssow, 2012). Here we aim to review the existing literature on *C. difficile* phages and to highlight the pros and cons, challenges and solutions, associated with developing them as a therapeutic.

C. difficile PHAGE THERAPY

The use of *C. difficile* phages as a treatment for CDI would involve orally giving patients a *C. difficile* specific phage preparation. The phages would attach to the receptors on the *C. difficile* cells, and following the phage DNA entering the cell, would undergo replication and ultimately lyse the bacterial cell, releasing phage virions to infect surrounding *C. difficile* cells. This infection and lysis process would be repeated until all the *C. difficile* cells are killed and the infection is cleared. The different stages of a lytic phage infection of *C. difficile* cells are illustrated in **Figure 1**.

There are several reasons why phage therapy would be particularly suited for treatment of CDI. One is that it offers select advantages over existing antibiotic treatments. They include the specific nature of the phage–bacterial interaction, which would avoid exacerbating the gut dysbiosis (disruption of gut microbiota) that can be associated with treatment of CDI (Peterfreund et al., 2012). Another advantage is the ability of phages to replicate in a self-limiting manner at an infection focus. Importantly, the nature and the physical location of CDI may make phage therapy a viable option, as there are likely to be relatively few problems relating to the delivery of phages to the colon. Often patients are infected by a single strain of *C. difficile* (Eyre et al., 2012) and therefore there is not a complex population of organisms to target. In summary, the clinical need to develop new treatments to combat *C. difficile* infection, combined with the problems of antibiotic associated gut dysbiosis, and the physical location and general clonality of the bacterium during infection makes a phage-based therapeutic appealing.

It is perhaps not surprising then that the development of therapeutic *C. difficile* phages has attracted both academic and commercial attention. However, in order for a phage-based therapeutic to be successfully developed for this species, there are several aspects of *C. difficile* bacterial and phage biology that need to be better understood. Considering the global importance of the pathogen, there has been little research on *C. difficile* phages compared to those which infect other pathogenic bacteria, and the information available on phages associated with *C. difficile* has been produced by relatively few research groups. The lack of published work in this area can be seen from a Pubmed search for terms "*C. difficile*" and "phage" which at the time of writing produced 45 results whereas "MRSA" and "phage" resulted in 342 publications. The lack of research in this area likely reflects the technical difficulties of working with anaerobes in general, and *C. difficile* in particular. However, existing research has provided key insights into the host–phage relationship for this species and for the prospects of using phage therapy against CDI.

SPECIFICITY-RELATED ADVANTAGES OF USING PHAGES FOR TREATMENT OF *C. difficile*

Clostridium difficile infection is characterized by a dysbiosis of the human gut microbiota (Manges et al., 2010). This imbalance results in the overgrowth of endogenous *C. difficile* (strains present in the person) or exogenous *C. difficile* (strains acquired from an external source; Eyre et al., 2012). Although mixed infections with multiple strains of *C. difficile* occur, they are thought to do so at a relatively low frequency, with the majority of infections caused by a single strain (Eyre et al., 2012). Healthcare associated epidemics have also been found to be dominated by single types (Loo, 2006; Hubert et al., 2007; He et al., 2012). The limited *C. difficile* strain types in individual patients means that as long as the appropriate phage is delivered, it is likely to be effective in clearing bacteria from infected individuals, and from sets of individuals who are infected during an outbreak setting.

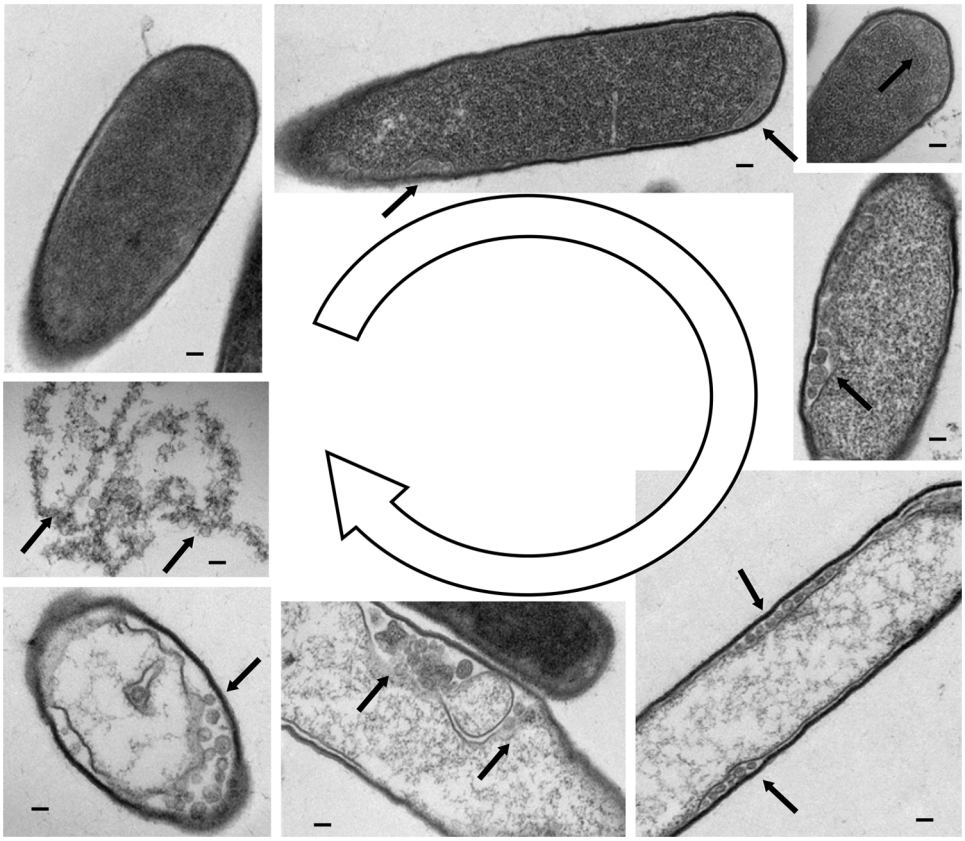

FIGURE 1 | Lysis of *C. difficile* cells by myovirus phiCDHM1. Transmission electron microscopy on microtomed samples showing various stages of the phage lytic cycle. Clockwise from top left, a cell with a single attached virion. Photomicrographs show an increasingly granular appearance inside the cell, and the formation of putative capsid structures at the outermost edges of the cell as indicated by arrows. The final image shows the contents of the lysed cell and multiple phage particles. Scale bars represent 10 nm. TEM photomicrographs courtesy of Katherine Hargreaves and Natalie Allcock, the Electron Microscopy Facility, University of Leicester.

The co-evolution of host bacteria and their predatory phages contributes toward the typically narrow host ranges reported for many phages (Hyman and Abedon, 2010) and it is this precise targeting of hosts that is exploited in the use of therapeutic phages (Kropinski, 2006). The phage mechanism of action is in contrast to the generally wide spectrum of activity that some antibiotics exhibit. This includes vancomycin which is commonly used to treat CDI and concomitantly exacerbates gut dysbiosis (Louie et al., 2012). Phage therapy would take advantage of the highly specific bacterial host ranges often exhibited by phages and therefore *C. difficile* would be removed, but commensal bacteria would be left intact.

Finally, another use of phage therapy would be not to replace antibiotics, but to extend the usefulness of current antibiotics, as the emergence of vancomycin and metronidazole resistance following treatment has been reported (Al-Nassir et al., 2008). Phages could be used as a first line of defense and antibiotics saved for a last resort.

ADVANTAGES OF *C. difficile* PHAGES TO TREAT BIOFILMS

Unlike antibiotics, phages are self-replicating. Once a susceptible host has been encountered and infected, phages replicate and the delivery of treatment is amplified locally. This quality is particularly desirable when targeting bacterial biofilms as they are notoriously difficult to penetrate and clear with antibiotics (Stewart and Costerton, 2001). Antibiotics have been shown *in vitro* to be ineffectual in clearing *C. difficile* biofilms (Dapa and Unnikrishnan, 2013). This is of clinical significance as *C. difficile* aggregates have been observed on the surface of caecum and colon tissues *in vivo* (Buckley et al., 2011). The successful use of phages to penetrate and disrupt biofilms has been reported in several species, for example in *Pseudomonas aeruginosa* (Hanlon et al., 2001; Fu et al., 2010), *Staphylococcus aureus* (Kelly et al., 2012), and *Campylobacter jejuni* (Siringan et al., 2011). The way in which phages degrade biofilms is often enzymatic with specific phages encoding enzymes that are effective for a target species (Flemming and Wingender, 2010). As *C. difficile* can form biofilms, it is possible that *C. difficile* phages will also have suitable specific enzymatic ability.

MINIMIZING PROBLEMS WITH PHAGE RESISTANCE

The continuous evolutionary dynamics played out between bacteria and phages has resulted in bacteria gaining multiple and diverse mechanisms to avoid and resist phage infection and predation

(Labrie et al., 2010). However, phages have co-evolved alongside their bacterial hosts and have counter-evolved strategies to maintain infectivity in what is often termed an "evolutionary arms race" (Stern and Sorek, 2011). Although evolved resistance to phages has not been reported in *C. difficile,* genome sequencing has revealed the presence of defense mechanisms including a CRISPR/Cas system (Sebaihia et al., 2006), and active type I and type II restriction modification systems (Purdy et al., 2002).

Another way in which bacteria can evolve resistance is to render the phage receptors ineffective. Although no phage receptors have been identified for *C. difficile* phages, one study observed that phages can adsorb onto diverse *C. difficile* isolates, but were unable to lyse them, suggesting that the receptor for the phage used in this instance is well conserved even across isolates that are not susceptible to lytic phage infection (Ramesh et al., 1999). In some Gram-positive bacteria, such as *S. aureus*, the wall teichoic acid has been identified as being essential for phage infection (Xia et al., 2011). Other candidate receptors for *C. difficile* include the S-layer that forms a paracrystaline layer around the whole bacterial cell and is highly variable between strains (Calabi et al., 2001). Although other sugars and proteins protrude through the S-layer, they are present in much lower abundance (Fagan and Fairweather, 2014). The S-layer has also been shown to be a receptor in other species such as the Gram-negative bacterium *Caulobacter crescentus* (Edwards and Smit, 1991).

Although the evolution of resistance to phages for therapeutic purposes is of genuine concern, there are strategies that can be used to minimize selection pressure for bacterial resistance. One such approach is to infect the target organism with a range of phages that have different receptors/modes of infection so changes in several targets would be required for phage resistance to emerge. Importantly, evolving phage resistance may not pose a significant clinical challenge as it can come at a cost to bacterial fitness or virulence (Koskella et al., 2012). This has been observed *in vivo,* in a phage therapy model of *C. jejuni* infections in chickens where emergent phage resistant mutants were not as competitive in growth assays as the non-phage resistant parent strain (Scott et al., 2007). The rates of phage resistance, or how they compare to antibiotic resistance, have not been determined for *C. difficile*, or indeed for many pathogens. However, the impact of phage infection on the development of antibiotic resistance has been investigated in *P. fluorescens* which showed that the application of phages did not accelerate antibiotic resistance (Zhang and Buckling, 2012).

THE EARLY YEARS OF *C. difficile* PHAGE RESEARCH; PHAGE TYPING

Having discussed the potential benefits of using *C. difficile* phages as a therapeutic, we now discuss research on *C. difficile* phages which has been conducted over the last three decades. They first became the subject of research attention following the realization of the pathogenic nature of the bacterium in the 1970s. **Figure 2** illustrates the progression of *C. difficile* phage research, showing a timeline of significant milestones that followed on from the discovery that *C. difficile* caused pseudomembranous colitis (George et al., 1978).

The lytic activity exhibited by *C. difficile* phages was initially exploited for typing purposes (Sell et al., 1983) and to provide information on the transmission and epidemiology of *C. difficile* strains (Hawkins et al., 1984; Bacon et al., 1988). The carriage of inducible prophages was also investigated in relation to bacterial phenotype and virulence, but did not establish a defined "lysotype" that reflected these phenotypic traits (Nagy and Foldes, 1991). Phage typing was ultimately not successful and its practice was discontinued, this was in part due to their narrow host ranges and strain typing was replaced by emerging molecular typing techniques (Dei, 1989).

THE EARLY YEARS OF *C. difficile* PHAGE RESEARCH; PHAGE THERAPY

The first *in vivo* model was developed using phage CD140 to treat clindamycin induced CDI in hamsters (Ramesh et al., 1999). This study demonstrated the potential usefulness of phages for CDI, with 14/18 hamsters surviving infection with phage treatment whereas none in the infected control group survived. In the model, a single phage dose of 10^8 PFU was administered immediately following challenging the hamsters with 10^3 CFU of *C. difficile*, in all phage treated animals. Two groups then had phage doses administered at 8 hourly intervals up to 48 and 72 h. The longevity of phage therapy protection was tested by re-challenging the surviving hamsters 2 weeks later with a second administration of 10^3 CFU of *C. difficile*, but no further phage treatment. *C. difficile* and phage counts were obtained from the cecal contents of deceased animals and *C. difficile* numbers in phage treated hamsters ranged from 1×10^3 CFU/g to 3×10^6 CFU/g. Phage were recovered from three of the infected hamsters; two having 10^2 PFU/g and one with 10^4 PFU/g recovered. Promisingly, the model showed that phage treatment could protect hamsters against *C. difficile*, that phages could survive in the hamster gut and that *C. difficile* isolates remained susceptible to phage lysis when tested *in vitro*. However, the phage application did not fully clear *C. difficile* and protection was not long lived. This is significant when considering the scope for continued release of *C. difficile* spores from treated patients into the environment and the potential for RCDI episodes, which is highly problematic in antibiotic treated patients (Johnson, 2009).

A CATALOG OF *C. difficile* PHAGES

The first reported isolation of *C. difficile* phages was in 1983, for the phages described above that were used for typing purposes (Sell et al., 1983). Since then several phages have been described in the literature (Hawkins et al., 1984; Mahony et al., 1985, 1991; Bacon et al., 1988; Ramesh et al., 1999; Goh et al., 2005a,b; Sekulovic et al., 2014). The levels of characterization vary between the studies and include one or several of the following types of data; host range studies, growth dynamics, morphological information, whole genome sequences, and comparative genetics.

The phages from the studies listed above are mainly temperate that were replicated on permissive hosts following either their induction or spontaneous release from a bacterial lysogen. In contrast, some phages were found as "free agents" in sample

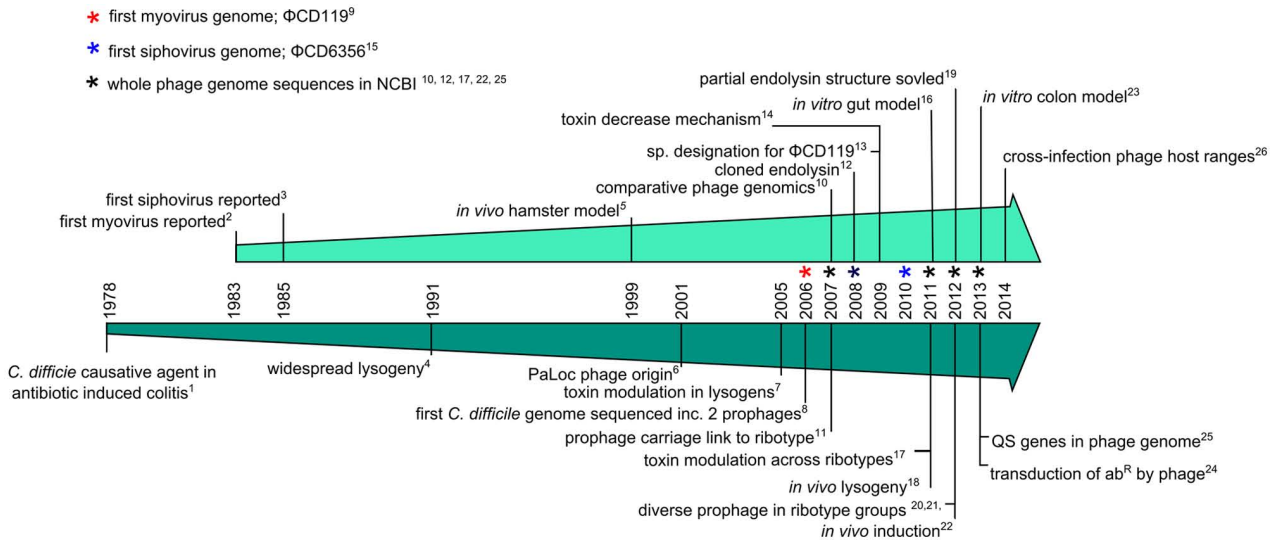

FIGURE 2 | Timeline charting major events of *C. difficile* phage research. Milestones are discussed in detail in the text and the references are as follows; (1) George et al., 1978, (2) Sell et al., 1983, (3) Mahony et al., 1985, (4) Nagy and Foldes, 1991, (5) Ramesh et al., 1999, (6) Tan et al., 2001, (7) Goh et al., 2005a, (8) Sebaihia et al., 2006, (9) Govind et al., 2006, (10) Goh et al., 2007, (11) Fortier and Moineau, 2007, (12) Mayer et al., 2008, (13) Lavigne et al., 2009, (14) Govind et al., 2009, (15) Horgan et al., 2010, (16) Meader et al., 2010, (17) Sekulovic et al., 2011, (18) Govind et al., 2011, (19) Mayer et al., 2011, (20) Nale et al., 2012, (21) Shan et al., 2012, (22) Meessen-Pinard et al., 2012, (23) Meader et al., 2013, (24) Goh et al., 2013, (25) Hargreaves et al., 2013c, (26) Sekulovic et al., 2014.

supernatants. Regardless of their origin, in all cases where the phage genomes have been sequenced, putative integrase genes have been identified which suggests that they can access the lysogenic lifestyle. Indeed, investigations have shown that both environmental and clinical derived *C. difficile* strains carry a diverse and prevalent set of prophages (Nagy and Foldes, 1991; Fortier and Moineau, 2007; Nale et al., 2012; Shan et al., 2012; Hargreaves et al., 2013b; Sekulovic et al., 2014). Collectively, these studies have shown that although some specific prophages appear to be associated with specific ribotypes, for other groups there are high levels of prophage diversity.

DEVELOPMENT OF ARTIFICIAL PHAGE TREATMENT MODELS

Two recent studies have used a one-phage/strain model in to explore *C. difficile* phage-host interactions in *ex situ* model systems. The first involved studying their dynamics in a batch gut model (Meader et al., 2010) and the second in a multi-vessel model of the colon (Meader et al., 2013). The gut model was performed in batch fermentations in which remedial and prophylactic treatments were tested using ϕCD27 (Meader et al., 2010). Results from both approaches showed significant decreases of CFU, as well as reductions in the levels of toxins A and B relative to no phage treatment. In contrast to the previous hamster model, lower multiplicity of infections (MOIs) were used; 7 in one replicate and 10 in the remaining two replicates. These MOIs resulted in different CFU counts between replicates, and *C. difficile* was cleared from the prophylaxis experiments when an MO1 of 10 was used. PFU counts of ϕCD27 indicated that the phage did not replicate in the model without *C. difficile* present, and there was no reduction in gut bacterial numbers observed based on CFU counts. The effect of metronidazole was tested in the model and found to reduce commensal CFU counts, highlighting the potential to avoid exacerbating the gut dysbiosis by phage treatment over antibiotic therapy.

The second study used a colon model with three vessels to represent the proximal and distal colons (Meader et al., 2013). The phage ϕCD27 was applied daily over 35 days at an MOI of 10. At days 14–21 clindamycin was added to the vessels to produce conditions which would permit *C. difficile* overgrowth. Three replicates were performed, but results were ambiguous. In two of the three, no vegetative *C. difficile* were isolated, but spore counts increased significantly. The third replicate produced CFU and spore counts similar to those of the controls. However, in all three replicates, the toxin levels decreased which is similar to the results in the previous gut model. To determine the cause of the different outcomes between replicates, *C. difficile* recovered from the third were assessed for lysogeny. Nine of ten colonies were positive for ϕCD27 following Mitomycin C treatment and the authors suggest that a lysogen generated early during the experiment went on to predominate the vessel's *C. difficile* population. What caused this to occur in one replicate and not the others is not known, but could have been due to the composition of the donor gut microbiota. Lastly, the effect on the gut bacterial population was also assessed in this study, expanding on the previous work by using denaturing gradient gel electrophoresis (DGGE) to profile the bacterial community. This was in addition to culture based detection and confirmed through these methods that phage infection did not appear to alter the community structure. Thus, the colon model data illustrates both the potential of phage therapy for CDI to clear *C. difficile*, as well as identifying other factors that could impact phage treatment.

ISOLATION OF *C. difficile* PHAGES

Phages that infect *C. difficile* have been notoriously difficult to isolate and propagate. Despite the early establishment that multiple phage panels could lyse divergent *C. difficile* strains, studies have reported low frequencies of propagatable *C. difficile* temperate phages on alternative 'host' strains via the lytic cycle; 0% (Nale et al., 2012), 3.9% (Sell et al., 1983), 2.1% (Mahony et al., 1985), 3.3% (Mayer et al., 2008), 4.7% (Horgan et al., 2010). This is despite the large numbers of strains (25–94) being screened and used both as sources and as hosts for phages. However, two studies have reported higher rates of propagating phages, with 7.14% (Goh et al., 2005b), and 15.5% (Sekulovic et al., 2014). It is likely that strain relatedness could influence the cross-infection of *C. difficile* phages which may explain low rates of some studies, for example Nale et al. (2012) screened strains only belonging to R027. It is also another motivating factor to seek phages from non-clinical backgrounds.

Two independent research groups have reported unsuccessful attempts to isolate free phages from patient, animal and environmental samples despite the use of multiple hosts and approaches (Goh et al., 2005b; Nale et al., 2012; Shan et al., 2012). A third group used enrichment on 15 *C. difficile* isolates representing eight different ribotype groups, and although they did not find phage in sewage samples (30 samples), they isolated four distinct phages from pooled stool samples (Meessen-Pinard et al., 2012). The researchers went on to show that these were temperate phages which they also detected as prophages in *C. difficile* isolates from the same stool samples.

The difficulties in isolating phages that can propagate on/infect *C. difficile,* have been attributed to its ability to undergo sporulation, a process that may select for lysogenic infections over lytic infections (Goh et al., 2005b). However, virulent phages have been isolated for other spore forming bacteria [e.g., *Bacillus cereus* (El-Arabi et al., 2013)] as well as for other *Clostridium* species [e.g., *Clostridium perfringens* (Volozhantsev et al., 2012)]. In all studies, large scale screenings containing diverse strains appear to be necessary to detect lytically active phages, and in addition to strain relatedness, this may be explained by the high proportion and diversity of prophage carriage between isolates (e.g., Hargreaves et al., 2013b). *C. difficile* lysogens are presumably able to resist both superinfection and secondary infection by related phages (e.g., Goh et al., 2005b). This has implications for the prospects of phage therapy as these lysogens would require therapeutic preparations to encompass a wide enough diversity of phages to counter their presence.

Although virulent phages are considered the most suitable for phage therapy, Fortier and Sekulovic (2013) stated in their recent review that they are not necessarily problem-free as phage therapy candidates for this species, as strains can have numerous prophages and recombination could potentially occur with a virulent phage. Thus, it is both pragmatic and pertinent to work with the phages that exist and which demonstrate lytic activity on specific *C. difficile* strains, but it is clearly advantageous to develop phages for therapy that have minimal or preferably no temperate activity on their target strains.

INSIGHTS FROM LYSOGENY IN *C. difficile*; IMPACT ON HOST PHYSIOLOGY

Another aspect of phage research in *C. difficile* has been to determine their role in CDI and evolution of this pathogen (e.g., Nale et al., 2012; Hargreaves et al., 2013b). It has been found that several *C. difficile* phages can modulate toxin production during lysogeny (Goh et al., 2005a; Govind et al., 2009; Sekulovic et al., 2011). Further genomic sequencing of isolated phages has described novel phage types (Horgan et al., 2010; Meessen-Pinard et al., 2012) and has revealed surprising genetic features, such as a phage with homologs of the bacterial accessory gene regulatory (Agr) system (Hargreaves et al., 2013c).

Collectively, the research described in this section has significantly expanded our understanding of the potential impact of phage on *C. difficile* physiology and on the suitability for specific phages as therapeutic agents. Clearly when designing phages as a therapeutic product, it is necessary to consider the potential of lysogenic conversion, which is particularly important in *C. difficile* as lysogeny is common. Multiple phage insertion sites have been identified (e.g., Govind et al., 2006; Goh et al., 2007; Williams et al., 2013), and one phage, φCD38-2, does not insert into the bacterial chromosome, but replicates as a circular plasmid (Sekulovic et al., 2011). The instability of both natural and laboratory generated lysogens is well documented, with *C. difficile* cells found to spontaneously release phages (e.g., Mahony et al., 1985), to differentially release their multiple prophages depending on the antibiotic they are exposed to (Sekulovic et al., 2014) as well as following freezing for storage (Goh et al., 2005b). Further to this, Meessen-Pinard et al. (2012) found that artificially generated lysogens produced significantly more phages when exposed to quinolones than the wild-type lysogens did. The impact of sub-inhibitory concentrations of antibiotics on lysogen induction is of relevance as antibiotic treatment may alter the levels of horizontal gene transfer (HGT) in *C. difficile* populations after exposure to quinolones when in patients.

Therefore it is also important to determine how a phage that has a tendency to lysogenize might behave during application as a therapeutic and how it may influence the host bacterium. These effects were examined in the phage, ΦCD119, where lysogens were shown to reduce toxin production (Govind et al., 2009). This occurs via the action of the phage-encoded RepR, which can bind to the promoter regions of *tcdR*, the positive regulator of *tcdA* and *tcdB*. To assess the impact of lysogeny *in vivo* during CDI, the same phage was applied in the Ramesh et al. (1999) hamster model Govind et al. (2011). The results showed that the phage could lysogenize under the conditions present in the mammalian gut, as *C. difficile* isolates were recovered that were unsusceptible to infection with ΦCD119. Prophage integration was confirmed using southern blots and PCR assays. Although lysogeny is not desirable in a therapeutic model, all three of the hamsters treated with phage outlived the controls. The lysogens may have become attenuated as inferred by the decrease in toxin production by the lysogens during culture. However, the toxin levels *in vivo* were not presented and the authors state that they will further determine the attenuation of strains in their future work.

Additionally, studies have shown that the physiological effect of lysogeny varies according to phage or strains used (Goh et al., 2005a; Sekulovic et al., 2011). Goh et al. (2005a) used three phages to lysogenise different strains, and the resulting lysogens produced increased levels of toxin B (3/5 lysogens) and toxin A (1/5 lysogens). Different strains lysogenized with the same phage however, differed in their toxin levels suggesting there is a strain-phage specific interplay that determines toxin levels. Sekulovic et al. (2011) found that two out of five generated φCD38-2 lysogens also stimulated toxin production. Interestingly, there was variation between isolates of the same ribotype. Together, these studies demonstrate that there is considerable variation in the physiological response of phage infection. Further work to establish the underlying mechanisms influencing toxin production in lysogens is needed to fully assess the potential impact it could have on phage therapy in this system.

INSIGHTS FROM LYSOGENY IN *C. difficile*; HORIZONTAL GENE TRANSFER

In addition to the introduction of novel genetic material in the form of the prophage genome, phage infection also presents the opportunity for generalized transduction to occur. The ability of *C. difficile* phages to mediate horizontal transfer of genetic material in this manner is possible, but has been little studied. While phage induced from a toxigenic strain did not convert a non-toxigenic strain following lysogenisation (Goh et al., 2005b), a recent study demonstrated that another phage, φC2, could mediate the exchange of novel genetic material between *C. difficile* strains (Goh et al., 2013). φC2 was shown to transduce an antibiotic marker *ermB* carried on a transposon 13 kbp in size, after infection with 10^7–10^8 PFUml^{-1} at MOIs of >0.02 (Goh et al., 2013). The size of the transposon is slightly smaller than the PaLoc which is ∼19.7 kbp (Cohen et al., 2000), and the length of DNA that can be transferred remains to be determined. It is known that *C. difficile* phages can access a greater number of strains than revealed by spot test assays alone, as demonstrated by the absorption of phage CD140 to multiple strains without producing lysis (Ramesh et al., 1999). Specific transducing phages could serve as useful molecular tools, as genetic manipulation of *C. difficile* has been difficult to achieve and there are relatively few methods, although a lethal vector system (O'Connor et al., 2006) and the ClosTron system (Heap et al., 2007) are available. Alternatively, phages able to access a broad range of strains without necessarily causing lysis could also be exploited for diagnostic purposes, as similar phage-based detection methods have been developed for other bacterial species such as fluorescently labeled phage, reporter phage or phage amplification assays [see review by (Rees and Dodd, 2006)]. More studies which further characterize phage host ranges are needed in order to assess the impact of transduction between *C. difficile* strains and how this may affect their application.

C. difficile PHAGE HOST RANGES

In general, data on host ranges shows that although some phages can lyse a range of *C. difficile* strains, typically host ranges are restricted to one or a few strains. In the few studies where ribotype information is presented, phages were found to infect across ribotype groups, for example, the phage ΦCD6356 can infect 13/37 strains which belong to five ribotypes (Horgan et al., 2010). The largest host range survey published showed that φCD38-2 could infect 99/207 isolates tested (Sekulovic et al., 2011). Although this seems high, 79 isolates belonged to the same ribotype group and in total only 11 ribotypes were assayed.

The use of phage panels in early typing studies showed that the inclusion of multiple phages resulted in the infection of many of the tested strains by at least one phage (Sell et al., 1983). As these studies predate ribotyping, no information is available about the diversity of the panel of strains being tested (Hawkins et al., 1984; Bacon et al., 1988). A recent study showed that distinct phages induced from different ribotypes, including φCD38-2 could lyse isolates belonging to several ribotypes of human and animal origins (Sekulovic et al., 2014).

The ability of phages to infect a broad range of *C. difficile* strains is considered to be ideal for therapeutic purposes, and this could be achieved by combining phages into a mixture or 'cocktail.' To date, only single phages and single *C. difficile* strains have been tested in models for treatment (Ramesh et al., 1999; Meader et al., 2010, 2013; Govind et al., 2011). Extensive host range analysis relating to genetic diversity of both strain and phages is currently being investigated in this laboratory (unpublished). The goal of this work is to determine the interactions between related phages and strains and to develop optimal phage mixtures to treat clinically relevant strains. Identifying phage receptors would help to inform these studies and introduce the possibility of altering phages to target a wider spectrum of strains. The mechanisms of phage resistance in this species also need to be addressed in order to establish the processes constraining the current narrow host ranges that have been reported.

EFFECT OF *C. difficile* PHAGES ON THE GUT MICROBIOTA

As previously highlighted, a key advantage that phages offer for CDI treatment is their specificity, both to reduce dysbiosis of the gut microbiota associated with CDI and to minimize the potential introduction of virulence genes from the pathogenic *C. difficile* to commensal organisms via transduction or lysogeny. In the gut and colon models, there was no detected impact on gut commensal communities resulting from phage treatment (Meader et al., 2010, 2013). Similarly, lysis of closely and less-closely related bacterial species has been examined in several studies (Sell et al., 1983; Ramesh et al., 1999; Goh et al., 2005b; Horgan et al., 2010). These species include an assortment of clostridial species including *C. sordellii, C. septicum, C. bifermentans, C. sphenoides,* and *C. perfringens* with no infective activity reported. However, some cross-infection has been observed as temperate phages from *C. sordellii* were found to be active on *C. difficile* (Schallehn, 1985). In addition, the genetic similarity reported between sequenced phages and other clostridial phages, such as PBSX (Govind et al., 2006), suggests that these phages could share an ancestry or infect across species. The impact *C. difficile* phages have upon patient's gut microbiota would benefit from the application of NGS technology to investigate the bacterial-phage and phage–phage dynamics during phage treatment. Bacterial and viral genomic and transcriptomic data would be useful for determining the

in situ population diversity and turnover of phages in order to elucidate the dynamics in this system. Functional data from transcriptomes and proteomes generated from infection models would also provide valuable insight into *C. difficile* phage–host interactions.

GENOMIC FEATURES OF THE *C. difficile* PHAGES

In this section of the review we discuss the insights into the interactions between *C. difficile* phages and their bacterial hosts gained from the study of their genomes. Many phage genomes are known to contain genes that can alter the physiology of the bacterial cell, including known toxin genes such as those carried by *Escherichia coli* STX phages and the toxin converting *C. botulinum* phage C1 [see review by (Boyd, 2012)]. In order to determine the suitability of specific phages for therapeutic use it is necessary to determine whether they carry genes that could enhance bacterial virulence. Genome analysis can also reveal the genetic relatedness between phages; this information can be used to inform the development of phage cocktails or be useful for other phage applications.

All *C. difficile* phage genomes sequenced to date are dsDNA and belong to the Caudovirales (the order of tailed phages). They can be grouped by particle morphology as they differ in size and morphological type which includes the long tailed myoviruses (LTMs) ϕCD27 and ΦMMP04, the medium myoviruses (MMs) ΦCD119, φC2 and phiCDHM1, the small myovirus (SMV) ΦMMP02 and two morphologically distinct siphoviruses (SVs) ΦCD6356 and φCD38-2. In this section we have performed a comparative genome analysis to highlight specific genome features of interest (**Figure 3**). The genome figure has been produced using EasyFig v.2.02 software (Sullivan et al., 2011) and subsequent analysis in Clustal Omega (Sievers et al., 2011).

While no close homologs of the *C. difficile* toxin genes are present in any of the phage genomes, Goh et al. (2007) identified a coding DNA sequence (CDS) in φC2 that has a low level of similarity to *tcdB* at the amino acid (aa) level. This CDS is located within a proposed lysogenic conversion module of the genome, flanked by the lysis and lysogeny control genes and contains CDSs primarily on the antisense strand. This region appears to be present in the other medium and LTMs, but the gene content and exact location of this region varies between phages.

Comparison of this region suggests that recombination between phages may have occurred within this module, as the genes appear to be in cassettes; for example the *tcdB*-like gene of φC2 is adjacent to a putative enzyme (as it contains a DUF955 protein domain, PFam E-value 1.2e-19, with a characteristic H-E-X-X-H motif at aa residues 80–84) and a CDS which encodes a predicted DNA directed RNA polymerase 7 kDa peptide/zinc finger protein [with a LIM domain, (PFam E value 0.066), but not a predicted transmembrane helix as assessed using the TMHMM server v. 2.0 accessed at http://www.cbs.dtu.dk/services/TMHMM/]. This gene cassette is conserved between φC2 and ϕCD27, with each CDS having 100% identity at the aa level between the two phages. The phage ΦMMP02 also encodes a homolog of the third CDS, which shares 100% identity with the genes of φC2 and ϕCD27, but does not have either of the other two CDSs. Also in ΦCD119 we detected an unannotated CDS on the antisense strand which contains a possible LIM protein domain (PFam E-value 0.062), but not the other genes in the cassette, which highlights the high degree of mosaicism between these related phage genomes.

The proposed lysogenic module of phiCDHM1 is notably different to the other sequenced phages. It carries a cassette containing gene homologs of the *agr* quorum sensing system. These genes are not shared with any of the other *C. difficile* phages. They represent a third type of *agr* locus present in relatively few *C. difficile* strains (Hargreaves et al., 2013c).

Another marked feature is the RepR of ΦCD119. Lysogenic infection with ΦCD119 has been found to reduce production of TcdA and TcdB, and RepR appears to repress transcription of *tcdA* and *tcdR* via binding of their promotor regions (Govind et al., 2009). A blastp search of this gene against all the annotated ORFs in each phage genome revealed homologs in phiCDHM1, φC2 and ΦCD6356; phiCDHM1_gp43 shares 30% identity with an E value of 2e-11, the gene phiC2p50 has 35% identity with an E value of 6e-18 and, lastly, ΦCD6356_38 has 27% identity and an E value of 2-10. Whether these genes have homologous functions also remains to be investigated but we can predict that these phages have genes with the capacity to influence cell physiology via regulatory proteins.

A key finding from the study that sequenced the genome of φC2 was that the PaLoc may have had a phage origin due to the sequence similarity between the holin gene and *tcdE* (Goh et al., 2007). This suggestion is further supported in the same study by the identification of a homolog of *cdu1,* Orf 46, which also contains a Penicillinase R protein domain. The CDS is located downstream of the lysogenic conversion module, on the sense strand, and may have a regulatory function. Homologs are present in ΦCD119, phiCDHM1 and ΦMMP02: phiCD119_gp42 has 48% identity and an E value of 1e-20, phiCDHM1 has two homologs; CDHM1_gp40 has 47% identity and an E value of 1e-31 and CDHM1_gp39 has 31% identity and an E value of 5e-19; ΦMMP02 also has two homologs; D863_gp39 has 31% identity and an E value of 2-19 and D863_gp40 has 44% identity and an E value of 5e-27. The functions of these genes are not known nor whether they would be the same for the compared genes.

Lastly, another CDS with a predicted accessory function has been identified in φC2, Orf37, which is a homolog of AbiF (Goh et al., 2007). AbiF is a protein involved in phage abortive infection [see review by Labrie et al. (2010)]. Homologs of this gene are not present in any of the other *C. difficile* phages and while Abi genes are typically encoded by their bacterial hosts, they have been found on a prophage element in *Lactococcus lactis* (Ventura et al., 2007) and in the genome of *L. lactis* phage TP712 (Roces et al., 2013). Although the mechanism by which AbiF performs its function is not fully known (Garvey et al., 1995), the unusual presence of this gene in the genome of phage φC2 suggests it could inhibit secondary phage infection or modulate φC2 replication and may contribute to the low frequencies reported for isolating free phages.

TAXONOMIC DIVERSITY OF THE *C. difficile* PHAGES

The relatedness of the sequenced phage genomes can be seen in **Figure 3** which includes their sequence similarity at the nucleotide

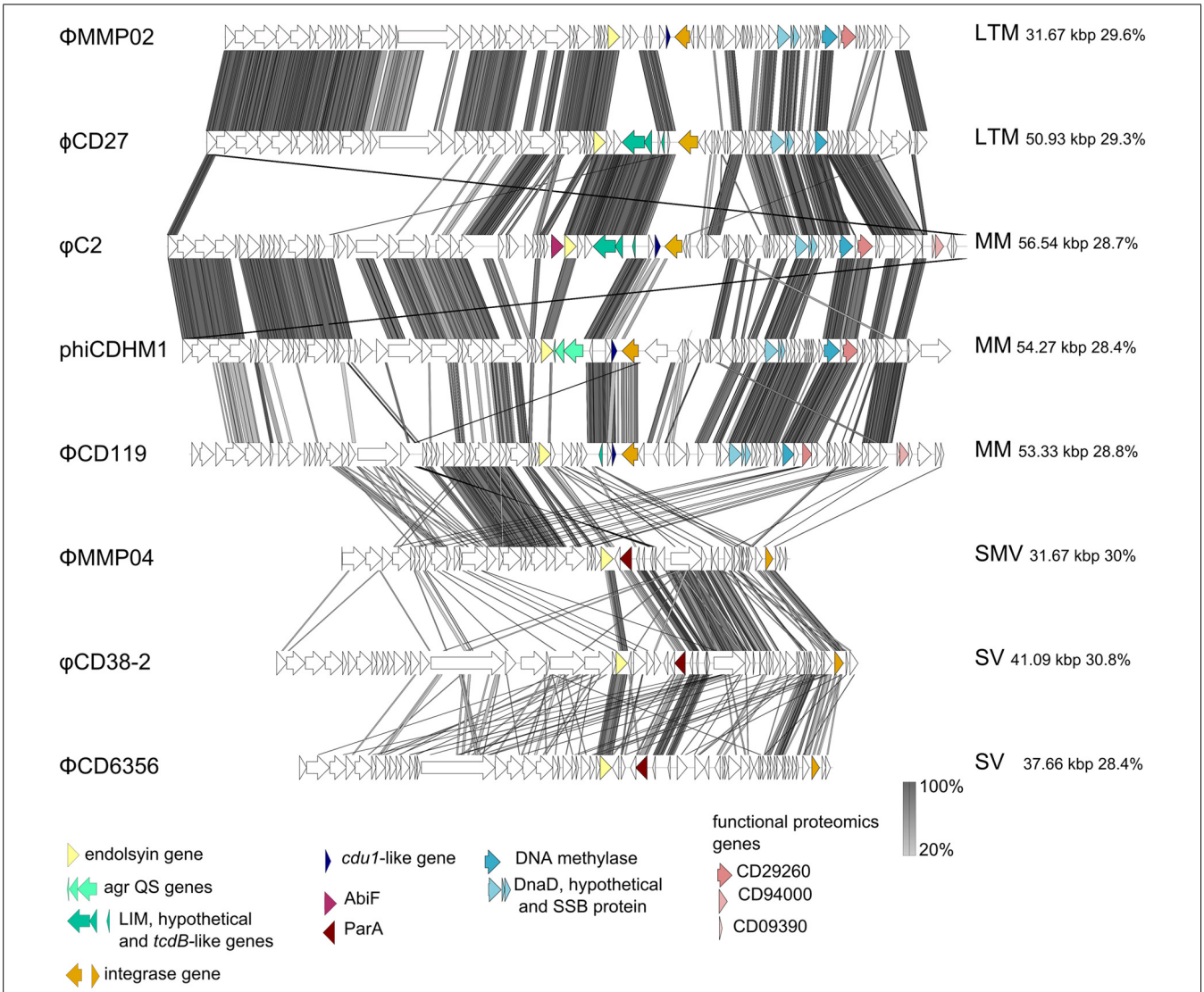

FIGURE 3 | Sequenced *C. difficile* phage genome similarity and content. Phage genomes were searched using tblastpx against one another and genome maps were generated using EasyFigv2.1 (Sullivan et al., 2011). The most similar phages are adjacent to one another, phage names are on the left hand side and phage morphological type, genome size and GC content are on the right hand side. The acronyms designate long tailed myovirus (LTM), medium myovirus (MM), small myovirus (SMV), and siphovirus (SV). Accession numbers for each phage genome are as follows: ΦMMP02 NC_019421.1; ϕCD27 NC_011398.1; φC2 NC_009231.1; phiCDHM1 HG531805; ΦCD119 NC_007917.1; ΦMMP04 NC_019422.1; φCD38-2 NC_015568.1; ΦCD6356 NC_015262.1. Specific genes are highlighted to shown their conservation and position between genomes, and are discussed in the text.

level, resulting from a tblastx analysis performed in EasyFig v.2.0 (Sullivan et al., 2011). The genome sizes vary from ∼31 to ∼56 kbp, but all share a similar GC content which ranges between ∼28–30%. In addition to differences in length, the genomes of the SMVs and the SVs are distinct in terms of their architecture compared to the medium and LTMs. The structural genes are followed by a lysis module, but in the genomes of the SMV and the SVs, the predicted integrases are located following DNA metabolism and DNA replication genes, on the sense strand. However, these phages do have putative lysogenic conversion modules, which are located immediately downstream of the lysis module in each genome. This region encodes predicted proteins with putative accessory functions including membrane or secreted proteins, and all three encode a homolog of ParA, a protein involved in partitioning DNA during cellular division (Bignell and Thomas, 2001). In the genome of ΦMMP04, there is a CDS, D864_gp28, which contains a flagella assembly protein domain, FliH (PFam E value 0.048), as well as a CDS, D864_gp30, which contains a MazE domain (PFam E value 0.025). This region in the two SVs is genetically similar, despite the fact that these phages are of different replication types, *cos* and *pac*, and that the regions containing structural genes are highly divergent to one another.

The subject of taxonomy within *C. difficile* myoviruses was addressed by Lavigne et al. (2009) in a review which predates the

publication of either *C. difficile* SV or the SMV genomes. In this review, the authors identified a DNA methylase gene and a DNA replication cassette which are characteristic for the type species ΦCD119. This cassette contains three genes that encode DnaD, a hypothetical protein and a single strand DNA binding protein. The DNA methylase gene and cassette are labeled in **Figure 3**. Although both of the SVs have a gene encoding a predicted single strand DNA binding protein, the other genes are not present in either the SV or SMV genomes and furthermore, these phages do not share 40% of their genes to the type species ΦCD119, or to one another, so could each represent a novel phage type species.

Notably these phage genomes contain divergent regions where genes predicted to encode tail proteins and tail fibers are located, suggesting that these phages could target divergent receptors. There is some similarity between the medium and SMVs in this region of their genomes, and in conserved genes located within it, but there is little to no similarity between the SVs at the aa sequence level. In order to determine whether sequence information can be used to guide phage selection for use in therapeutic cocktails, investigation into the tertiary structures of the putative tail fiber sequences as well as co-absorption assays may be helpful.

ALTERNATIVES TO THE USE OF "WHOLE PHAGE" TO TREAT CDI

The major disadvantages that have been described against the therapeutic use of *C. difficile* phages are their narrow host ranges and their ability to lysogenize strains of *C. difficile*. To combat these problems, one alternative approach for applying *C. difficile* phage biology in a therapeutic manner has been to clone and express a recombinant version of the endolysin from ϕCD27 (Mayer et al., 2008). Endolysins typically have a cell wall binding domain and an amidase domain, which degrades the bacterial peptidoglycan layer resulting in cell death and lysis. The endolysin has a wider host range than the phage it has been cloned from, which suggests the target of this enzyme in the peptidoglycan structure of different *C. difficile* strains is less variable than that of the phage receptor molecule (Mayer et al., 2008, 2011).

Although this is a non-replicating approach, the endolysin has been cloned into *L. lactis* to demonstrate a proof of principle for delivery to the gut and sites of infection (Mayer et al., 2008). The further investigation of this endolysin found that a truncated version of the N terminus was able to lyse all 32 strains of *C. difficile* tested, and lysed cells more rapidly and effectively than the intact enzyme (Mayer et al., 2011). Surviving cells grew more slowly, and presumably growth rate was subject to the cost of resistance or cell damage. However, the effective host range was also broader, as researchers found that strains of two other clostridial species were lysed by both the intact and partial endolysin, *C. sordellii* and *C. bifermentans*. The endolysin could also lyse less-closely related species, *B. amyloliquefaciens*, *B. cereus*, and *B. subtilis, Listeria innocua, L. monocytogenes serovar 4, L. ivanovii* NCTC 11007; and as the researchers state, this may be explained by the fact that all have a peptidoglycan type of A1γ. How this wider activity of the truncated endolysin impacts upon on the microbial community of the GI tract remains to be tested.

FUTURE DIRECTIONS

As discussed in this review, there are several potential benefits of using phage therapy to treat patients with CDI. Studies have shown both the efficacy of phages to clear and/or prevent infection and demonstrated the specificity of phages for targeting *C. difficile* in the gut microbial community. However, research into the biology of these phages has also demonstrated a high frequency of lysogeny by known phages and it has highlighted the fact that phages may influence *C. difficile* physiology when present as prophages, or when infecting in the lytic cycle. These different aspects of *C. difficile* phage biology need to be addressed during the development of any therapeutic using such phages. Lysogeny is also undesirable due to the potential transfer of novel genetic material into the recipient cell, and may make the bacterial cell resistant to the phage therapy. This would mean that if the lysogen was spread through a population, the cells that encoded it could not be killed by the same phage that was being given as a therapeutic.

These considerations motivate the continued attempts to isolate strictly virulent phages, but there are also alternative strategies which make use of existing phages. The emerging technology of synthetic biology to alter phage host ranges or to synthesize a strictly lytic phage by mutating a temperate phage is theoretically possible. Such procedures have been achieved for other systems and purposes, e.g., for enhancing antibiotic susceptibility of the host bacterium (Lu and Collins, 2009). Furthermore, synthesizing modified phages has been investigated for a number of pathogenic bacteria such as *E. coli* and *S. aureus* [see review by Moradpour and Ghasemian (2011)]. Genetic manipulation of the phage hosts could also be performed to improve phage isolation or production, as has been done in other bacterial species, for example the removal of prophage elements in *Corynebacterium glutamicum* (Baumgart et al., 2013).

It is worth noting however, that the use of genetically modified phages and indicator hosts could present further difficulties to regulate their use. To avoid the use of GMOs, optimization of existing techniques could be performed, such as continued passage on specific strains to improve host ranges, as has been demonstrated for *P. aeruginosa* (Betts et al., 2013), as well as to devise new approaches, such as using lysogens generated from known phages to screen for less related phages. Additionally, the characterization of host ranges to include more determinant features as suggested by Hyman and Abedon (2010) would aid in understanding the biological parameters in this system.

Also, as yet, no experimental models have included the investigation of multiple phages or strains, and the application of phage cocktails both in *in vitro* and *in vivo* models would help determine their efficacy against a range of *C. difficile* strains. Such experiments could show how resistance could be countered, as well as establish the significance of lysogeny within a multi-phage approach. Such work is currently underway in this laboratory (UoL, 2013). Another option is to expand the models available to assess phage therapy, for example the use of *ex vivo* models such as human epithelial cell lines is being researched in our laboratory (unpublished data, this laboratory) as well as mathematical modeling to assess host range data (unpublished data, this laboratory).

Concurrently, research into how to deliver phages and their production is also required. Phage particles have been found to be inherently stable under specific conditions (e.g., Hargreaves et al., 2013a) and studies of *C. difficile* phages have found they can be stable across a range of pHs and temperatures (Sell et al., 1983; Mayer et al., 2008; Horgan et al., 2010), but stability is variable according to different phages (Mahony et al., 1985), and the use of a bicarbonate buffer administered in an *in vivo* model was necessary for phage viability (Ramesh et al., 1999). Problems with obtaining high titres of phage has also been addressed and researchers have optimized phage production by including divalent cations to infected cultures, altering agar concentrations and by infecting cultures at varying bacterial growth stages which have resulted in phage titres as high as 10^{10} PFU/ml (Mahony et al., 1985; Goh et al., 2005b; Horgan et al., 2010).

Aside from developing the production of a phage therapeutic, further work is needed to establish the consequences of phage infection on both the host bacterium and on the neighboring microbial community. For example RNAseq data could be used to generate transcriptomes, as has been done in other systems, such as in a *Pseudomonas* phage infection model by (Lavigne et al., 2013). In the same study, the authors also utilized proteomic data to probe infection dynamics, highlighting the usefulness of combining several methods of analysis to gain an accurate insight into the temporal dynamics of phage infection. There are few published studies examining the *C. difficile* proteome (e.g., Chen et al., 2013), but they offer valuable data and one such study revealed the presence of phage proteins in CD630 spores (Lawley et al., 2009). Homologs of these genes are highlighted in **Figure 3**, but how phage infection impacts upon sporulation processes is not understood in this species, although it has been well described in another endospore former, *B. anthracis* (Schuch and Fischetti, 2009).

Next generation sequencing has also been applied to aid understanding of the mechanisms facilitating HGT in the normal human microbiota (e.g., Smillie et al., 2011). In *C. difficile*, the PaLoc can be transferred via conjugation as shown during *in vitro* experiments (Brouwer et al., 2013). Understanding the levels of HGT occurring during CDI would be useful in order to determine how this could be impacted upon during phage treatment. It is known that antibiotic treatment can modify the interactions between phage and bacteria (Modi et al., 2013), but this has not been examined for *C. difficile* treatment models including antibody and antibiotic treatment (e.g., Peterfreund et al., 2012) and bacteriocin treatment (Rea et al., 2011). As previously discussed, more information on the bacterial and phage dynamics during CDI would be helpful to assess the impact on prescribing phages as therapeutics.

Research on *C. difficile* phages has revealed key insights into the evolution of this pathogenic bacterium as well as providing resources that can be exploited in many ways. This includes the use of transducing phages, of phages as diagnostic agents, as sources of therapeutic proteins, and indeed as therapeutic agents themselves. To conclude, we concur that *C. difficile* phages are indeed still difficult. They are technically demanding to isolate and propagate and several aspects of their relationship with their bacterial hosts are still unclear. However, their potential value as therapeutics and the emergence of the new sequencing and molecular tools available to researchers should provide answers to the questions which underpin the successful development of a phage-based therapeutic.

ACKNOWLEDGMENTS

We would like to thank the phage community, particularly those who frequent Evergreen Phage Meetings, for their encouragement, useful discussions and advice over the years that have helped direct our efforts to understand *C. difficile* phage biology.

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Conflict of Interest Statement: We have strived to write a fair and balanced view of the potential to develop *C. difficile* phages for therapeutic purposes. The work discussed in this paper was supported by funding from an MRC New Investigator Award to Martha R. J. Clokie G0700855. Martha R. J. Clokie's laboratory currently receives funding from AmpliPhi Biosciences Corporation, Glen Allen, VA, USA, to facilitate the development of a set of *C. difficile* phages for therapeutic purposes although data from this work is not included here.

Received: 31 January 2014; accepted: 03 April 2014; published online: 28 April 2014.
Citation: Hargreaves KR and Clokie MRJ (2014) Clostridium difficile phages: still difficult? Front. Microbiol. ***5****:184. doi: 10.3389/fmicb.2014.00184*
This article was submitted to Virology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 18 February 2014
doi: 10.3389/fmicb.2014.00051

The factors affecting effectiveness of treatment in phages therapy

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In recent years, the use of lytic bacteriophages as antimicrobial agents controlling pathogenic bacteria has appeared as a promising new alternative strategy in the face of growing antibiotic resistance which has caused problems in many fields including medicine, veterinary medicine, and aquaculture. The use of bacteriophages has numerous advantages over traditional antimicrobials. The effectiveness of phage applications in fighting against pathogenic bacteria depends on several factors such as the bacteriophages/target bacteria ratio, the mode and moment of treatment, environmental conditions (pH, temperature...), the neutralization of phage and accessibility to target bacteria, amongst others. This report presents these factors and the challenges involved in developing phage therapy applications.

Keywords: phage therapy, antibiotic resistance, effectiveness, pathogenic bacteria, Phage

INTRODUCTION

Bacteriophages (phages) are viruses that only infect bacteria. They are approximately 50 times smaller than bacteria (20–200 nm) and ubiquitous in the soil, water, and several food products (meat, vegetables, dairy products...; Kutter and Sulakvelidze, 2005). The "virulent" and "temperate" phages differ in their mode of action. The first step of the phage infection is adsorption of the phage particle to the bacterial cell wall by specific interactions between viral surface proteins and host cell receptors. After entering the bacterial cell, the virulent phages replicate rapidly to synthesize genome and structural proteins into progeny virions inside the host cell. Finally the new phages escape by rupturing the cell wall which results in the death of the cell. In contrast, temperate phages integrate their genetic material into the chromosome of the host cell, which is replicated along with the host cell genome (prophage). They can, therefore, subsequently emerge inside a new host cell. Only temperate phages which can enter the bacterial genome participate in horizontal gene transfers between bacterial populations. For antibacterial applications, virulent phages which have the ability to rapidly lyse bacterial cells are employed.

The use of bacteriophages to treat bacterial infections was studied prior to the Second World War. These studies were not followed up once antibiotics had been discovered. But in recent years, with the emergence of several bacterial strains multiresistant to antibiotics, research has turned back to bacteriophages. The use of phages to inactivate pathogenic bacteria is seen as an interesting way of replacing antibiotics in human medicine. Indeed, bacteriophages are considered as "intelligent antimicrobials" due to the specificity of their action. They infect the target bacteria without any effect on commensal flora and are naturally eliminated along with the complete eradication of pathogenic bacteria (Kutateladze and Adamia, 2010; Jenny, 2011).

In veterinary medicine, numerous studies have been carried out to combat bacterial diseases and control transmission to humans of the pathogens responsible for foodborne illnesses. For example, the reduction of *Campylobacter* and other pathogenic bacteria contamination by phages has been studied in several publications (Berchieri et al., 1991; Goode et al., 2003; Connerton et al., 2004; Fiorentin et al., 2005; Wagenaar et al., 2005; Higgins et al., 2008). The poultry and medical fields can benefit from these results, in terms of reducing economic losses and improving the overall well-being of consumers.

Bacteriophages have been studied in the agri-food industry in order to detect and control pathogenic bacteria in foodstuffs (Martínez et al., 2008; Guenther et al., 2009; Holck and Berg, 2009; Jamalludeen et al., 2009; Kocharunchitt et al., 2009; Hudson et al., 2010; Bigot et al., 2011; Mahony et al., 2011). The advantage of using lytic phages lies in their high specificity toward the host pathogenic bacterial strains. They do not affect technological flora or the commensal flora of the digestive tract. In addition, bacteriophages do not cause human allergies and nor do they change the structure, odor or flavor of food products (Hagens and Offerhaus, 2008).

Research on the phages has been extensive over the last decade (Pirnay et al., 2012). Many animal models have been available for reliable studies. Research on phages has expanded beyond the laboratory. Phage products have been approved and commercialized. Regularly, approvals have been granted in the USA for commercial phage products. In 2006 the FDA (Food and Drug Administration) approved the use and the preparation of bacteriophages generally recognized as safe (GRAS) as food additives for the control of the pathogenic bacterium *L. monocytogenes* in meat and poultry products. In Europe, the use of Listex^{TM} was also approved in Switzerland for cheese making and has recently been approved for other food types. The Listex^{TM} has also been approved for use in food processing by food standards Australia and New Zealand (FSANZ) in 2012. Several phage products are currently produced on a commercial scale:

BACTERIOPHAGES ON A COMMERCIAL SCALE

LMP-102^{TM} (Listshield^{TM}) produced by Intralytix Inc. (USA) includes six bacteriophages This product occurs naturally in the environment and is used to control *L. monocytogenes* in ready-to-eat meat and poultry products before packaging. Other similar products such as ECP-100^{TM} (Ecoshield^{TM}) target *E. coli O157:H7* in ground beef, fruits, and vegetables.

Listex^{TM} is a phage preparation derived from phage P100 that targets *L. monocytogenes* by EBI Food Safety (Netherlands). Phage P100 was originally isolated from a wastewater sample taken from a dairy plant in Germany in 1997 (Carlton et al., 2005). In 2011 the company started selling its new phage product, effective against *Salmonella*, branded: SALMONELEX^{TM}.

Omnilytics, Inc. (USA) has two products that target bacteria on animal hides prior to slaughter. Both products are termed BacWash^{TM} targeting *Salmonella* and *E. coli O157:H7*. BacWash^{TM} can be applied as a wash, mist or spray directly to the live animal. Future potential uses of the BacWash^{TM} line of products include the treatment of animal holding areas, transportation equipment and containers, and living areas.

Other phage products are currently commercialized and developed by several companies such as AgriPhage ^{TM} of Omnilyics (USA) targets *Xanthomonas campestris* or *Pseudomonas syringae*. BioPhage-PA is product of AmpliPhi biosciences Corp (UK) for the treatment against *Pseudomonas aeruginosa* in chronic ear disease and topical injection. Viridax ^{TM} is being developed to treat the *S. aureus* by Viridax Company (USA).

Despite this increase in research interest and the production of phage products, the application in phage therapy was not always successful. The effectiveness of phage applications against pathogenic bacteria depends on several factors such as the bacteriophage/bacteria ratio, physico-chemical factors (pH, temperature...), phage neutralization or resistance to phage. Moreover, the data *in vitro* cannot be directly applied to the *in vivo* situation and nor can *in vivo* data for one phage be transferred to another phage. Critical parameters that affect phage therapy are the phage adsorption rate, burst size, the latent period and initial phage dose (Payne et al., 2000; Payne and Jansen, 2001). Another critical parameter is the clearance rate of the phage particles from the body fluids by the reticuloendothelial system or the phage-neutralizing antibody phenomena. Some key factors are presented in **Figure 1**.

Phages/bacteria ratio

The use of bacteriophages against pathogenic bacteria has been studied using two different approaches, one passive, the other active (Gill, 2010). In the case of the passive approach, the bacteriophages are added into the system at a level sufficient to ensure that all target bacteria are infected and lysed in a short period of time. On the contrary, active biocontrol relies on the addition of a small amount of phages. Bacterial elimination, in this case, supposes the replication of phages over several generations. The capacity of new replicated phages to access the target bacteria could be weakened by the biochemical and physico-chemical characteristics of the system (the viscosity for example). It appears that the passive treatment is more efficient than the active one.

In virology the bacteriophages/bacteria ratio is explained by the term MOI (multiplicity of infection) which refers to the number of virus that are added per cell during infection. MOI is used only in fluid systems with high numbers of host cells. In *in vitro* and *in vivo* experiments on phage against bacteria, the MOI comprised between 0.01 and 100 are classically used. Most often, MOI is 100 to ensure there is enough phage in the media. However, not all phages replicate or survive in the same way. It is important to determine the replication lytic cycles and the resistance of phages in respect of environmental conditions.

Environmental conditions and phage resistance

The survival and persistence of bacteriophages are affected by physico-chemical factors (pH, ions, temperature...; Jonczyk et al., 2011). The phage population is generally stable in relation to external factors. Some phages can be stored for a long period in neutral pH (6 to 8) in solution or in dried form (Jonczyk et al., 2011). Phage titers generally are decreased slowly with pH. For example, the phage titer of *S. aureus* was reduced 2 log between 4 and 6 h when pH decreased from 6.19 to 5.38 (Garcia et al., 2009). The proliferation of several phages is limited when pH is lower than 4.5, but the risk of pathogenic bacteria food contamination is also generally reduced below pH 4.5. For example, the phage T4 (*Myoviridae* family) is unstable at pH < 5. Phage PM2 (*Corticoviridae* family) loses completly activity after 1 h at pH 5.0 at 37°C. However, in the case of phage oral injection, stomach acid can have a negative impact on the survival of phage which may lead to treatment failure (Watanabe et al., 2007). The latent period is increased when the phages are incubated at refrigeration temperatures. Bacteriophage can survive at high temperatures (40–90°C) and some phages of *Lactococcus* can survive pasteurization (Madera et al., 2004). In the study of MS2 phage stability in different salt solutions the authors showed that the monovalent salts did not influence phage titer (Mylon et al., 2010). According to Langlet et al. (2008) higher ionic strength can increase the aggregation of phage (Langlet et al., 2008).

Besides these environmental conditions, the biochemical composition of the matrix also influences the accessibility of target bacteria.

Accessibility to target bacteria

According to Marco et al. (2010) the diffusion of bacteriophages could be impaired or favored depending on the structure and the composition of the matrix and the environmental conditions (Marco et al., 2010). In solid media, the diffusion of bacteriophages could be limited, reducing phage adsorption on bacteria and, consequently, the phage infection capacity. For example, Guenther et al. (2009) have shown that the use of bacteriophages was limited by their diffusion in solid food matrices such as hot dogs, smoked salmon and seafood.

The presence of other compounds could protect bacteria from phages. The phage K is active on numerous strains of *S. aureus,* but was inactive in raw milk which limits its application in bovine mastitis (Gill et al., 2006a). O'Flaherty et al. (2005) suggested that the immune factors present in raw milk prevented phages from gaining access to bacteria. According to Gill et al. (2006b), some proteins in whey may inhibit the adsorption of phage on bacteria.

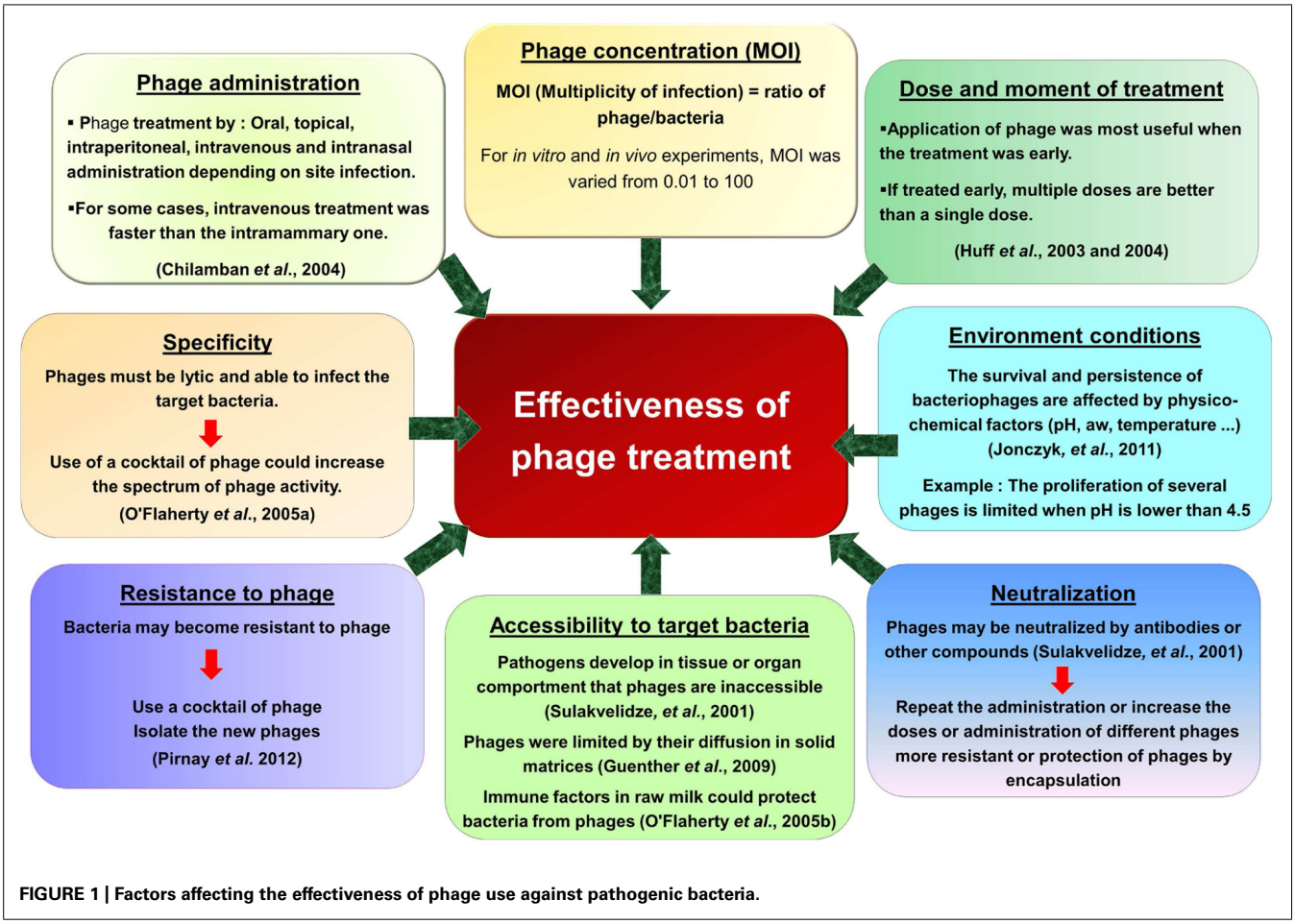

FIGURE 1 | Factors affecting the effectiveness of phage use against pathogenic bacteria.

In phage therapy, the escape of invasive pathogens into closed tissue and organ compartments may block the effective use of bacteriophages, especially if the phage cannot actively follow the bacteria. It is also unclear how effective phages would be in treating diseases caused by intracellular pathogens (e.g., *Salmonella* species), where bacteria multiply primarily inside human cells and are inaccessible to phages (Sulakvelidze, 2005).

In the study of Chibani-Chennoufi et al. (2004) the author showed that the survival of the bacteria in the gut during the phage passage could only be explained by some physiological reasons that prevented phage-induced lysis. The axenic mice were infected with a single *E. coli* strain and then were given phages in drinking water. The phage titers in the stools increased in one day from the 10^5/mL in the drinking water to 10^{10}/ml in the stool while at the same time numbers of *E. coli* in the stools reduced from 10^8 to 10^4 CFU/mL and stabilized at 10^5 CFU/mL during the subsequent days. The bacteria were not completely lysed in the stool although these bacteria are sensitive to the phages. These results suggest that bacteria had resided in gut sites protected from phage (Chibani-Chennoufi et al., 2004).

Phages, unlike many antibiotic molecules, are not diffusible across membranes and must therefore have a method of delivery to reach the target cells. Some researchers believe that the best delivery mechanism may lie in using other non-pathogenic species of bacteria to bring the phage to its pathogenic target (Inal, 2003).

Circulation of phage and neutralization of phage by antibodies

To understand further the accessibility of phage to bacteria, some authors have studied how phages circulate but few publications are available on the subject. Some authors suggest that phages get into the bloodstream of laboratory animals (after a single oral dose) within 2 to 4 h and that they are found in the internal organs (liver, spleen, kidney, etc.) in approximately 10 h. Also, data concerning the persistence of administered phages indicate that phages can remain in the human body for relatively prolonged periods of time, i.e., up to several days (Bogovazova et al., 1991, 1992).

In an experimental design where mice were infected with the ϕMR11 lysogen strain, no protection was observed allowing the authors to conclude that a direct bactericidal effect of the phage was the principal determinant of the protective effect rather than any indirect effect such as a phage-stimulated immune response (e.g., production of cytokines; Matsuzaki et al., 2003). Phage and bacterial numbers in the circulation were determined after the infection and showed that the bacterial load was much lower in the blood of phage-treated mice when compared to those that received only bacteria. They also noticed that phage titers

in mice infected with bacteria remained higher than the titers in mice that received only the phage. This suggested that the phage had replicated in the infected mice and consumed the bacteria.

Although the phage can circulate well in the blood and in different organs but some authors suggest that the phages may be neutralized by antibodies which hamper phage effectiveness to lyse the targeted bacteria. Geller et al. (1998) have showed that the addition of colostrum in milk contaminated by phage prevented the lysis of starter cultures of *L. lactis*. However, it is not clear how long the antibodies will remain in circulation. According to (Sulakvelidze et al., 2001), the development of neutralizing antibodies should not be a significant obstacle during the initial treatment of acute infections, because the kinetics of phage action are much faster than the host's production of neutralizing antibodies. Moreover, if phage-neutralizing antibodies are still present at the time the second course of treatment is administered or if a rapid anamnestic immune response occurs before the phages exert their action, it could be envisaged to repeat administration or increase the phage concentration. Another solution to survive the phage neutralization by antibodies would be to use different phages because resistance is different from one phage to another.

Merril et al. (1996) developed an ingenious method to solve this problem. They succeeded in isolating the mutants, whose stability in the blood increased, by repeating the following procedure eight to ten times: (1) administration of phages into the peritoneal cavity of the mouse, (2) recovery of phages from the blood 7–18 h after the injection, (3) multiplication of the recovered phages *in vitro*, and (4) readministration of the proliferated phages to mice. The mutant derived from phages after a long-circulating had capsid protein modified (Merril et al., 1996).

Protection of phages

One of the solutions to protect the bacteriophage at the site of infection and during the journey to this site is microencapsulation which is defined as a technology of packaging solids, liquids, or gaseous materials in miniature capsules that can release their contents at controlled rates under specific conditions. Microencapsulation has been applied to enhance the viability of probiotic bacteria during processing and also for targeted delivery to the gastrointestinal tract. The microencapsulation of viruses has been studied as an effective adjuvant system to induce specific immune responses via mucosal routes. Development of oral microencapsulated forms for bacteriophages to treat gastro infection in cattle has been reported (Ma et al., 2008; Dini et al., 2012). These authors have shown that the encapsulation technique enables a large proportion of bacteriophage to remain bioactive in a simulated gastrointestinal tract environment, which indicates that these microspheres may facilitate delivery of therapeutic phage to the gut.

Dose and moment of treatment

Another important factor that can modify the effectiveness of phage treatment is single dose versus multiple doses. Several studies have shown that multiple doses are better than a single dose. One study by Huff et al. (2003a,b) found that treating chickens suffering from severe respiratory infections caused by *E. coli* was very helpful in clearing up symptoms. The application of bacteriophage was most useful very soon after the chickens had been exposed to the bacteria and that, if treated early, multiple doses were better than a single dose. Interestingly, if treatment starts later, there is no difference between single or multiple doses, but treatment is still very helpful (Huff et al., 2003a,b).

In a very thorough study, Biswas et al. (2002) performed experiments using a mouse model of vancomycin- resistant *Enterococcus faecium* infection. They showed first that a phage administered intraperitoneally 45 min post-infection was able to rescue mice from *E. faecium* and that the rescue was associated with a significant decrease in bacterial numbers in the blood. They also demonstrated that phage administration up to 5 h post-infection still fully rescued the mice while treatment delayed beyond 5 h rescued only some of the mice (Biswas et al., 2002).

Phage administration

One advantage of phage use is the easy administration. Phages can be applied by oral, topical, intraperitoneal, intravenous, or intranasal administration. The phage preparation and production may be carried out in pathogenic bacterial culture. In this case it must be controlling the toxin or the residues of bacteria culture which may provoke the inflammatory phenomenon (Gill and Hyman, 2010).

Phage therapy has been used for the treatment of a variety of bacterial infections. They can be used in freeze-dried form and turned into pills without materially impacting efficiency. Temperature stability up to 55°C and shelf lives of 14 months have been shown for some types of phages in pill form. Application in liquid form is also possible, stored preferably in refrigerated vials.

Oral administration works better when an antacid is included, as this increases the number of phages surviving passage through the stomach.

Topical administration often involves application to gauzes that are laid on the area to be treated.

In the study of efficacy of bacteriophages for the treatment of infections caused by *Klebsiella ozaenae, K. rhinoscleromatis scleromatis* and *K. pneumonia* (Bogovazova et al., 1991, 1992), the phage preparation was reported to be efficacious in treating experimental infections of mice and guinea pigs. *Klebsiella* polyvalent bacteriophage was administered intraperitoneally, intravenously or intranasally on day 2 after the infection of the animals with *Klebsiella*. The result showed that the bacteriophage introduced intraperitoneally, was effective in the treatment of a generalized *Klebsiella* infection. Other authors Chilamban et al. (2004) have also shown that intravenous inoculation was faster than the intramammary one when they studied the efficacy of a specific lytic phage against *S. aureus* in a mice model.

However, it is difficult to conclude which mode of administration is the most effective. The effectiveness of treatment depends on various factors: the concentration of pathogenic bacteria on the infection site, phage preparation, and the dose applied, medium composition and structure, and environmental conditions...

Specificity

Phages specifically infect the host bacteria species. This specificity can limit the effectiveness of phage use. To ensure that the bacteria

can be lysed by the phage used, the bacterial strain isolated from the infection site will be tested for its sensitivity to the phage administrated. It is important also to verify if the phage is strongly lytic or not. However, polyvalent phages which can infect several bacteria strains of the same species do exist. The use of polyvalent phage allows the activity spectrum of phages to be increased. The polyvalent phage can be replaced by a cocktail of phages. Briefly, the phage by their specificity can infect only the target bacteria without effect on others bacteria flora, but the specificity may also have an ineffective treatment if the target bacteria are not lysed by the phages administrated. To overcome the problem related to the specificity of phage, several solutions are proposed:

- Isolating the bacteria from the infection site and screening the sensitivity of this bacteria against a panel of bacteriophages.
- Selecting polyvalent bacteriophages with broad cross-strain lytic activity
- Developing a cocktail of phages which could increase the spectrum of activity of the phages against all or most of the strains within a given species of bacterial pathogen.

Resistance to phage

As in the case of antibiotics, bacteria can develop resistance to phage, which may hamper the effectiveness of phage treatment. The first step of phage infection to bacteria is adhesion of phage on bacterial surface by surface proteins which act as receptors. If the bacterium loses the phage receptor, they become resistant to phage. Bacteria may also acquire horizontally a restriction-modification system that degrades the nucleic acid of the injected phage. In addition, phage resistance may be caused by a mutation in a gene, the product of which is essential for phage replication or assembly. In any case, the resistance to phage does not cause a problem for phage use or phage therapy because the rate at which bacteria develop resistance to phages is approximately 10-fold lower than to antibiotics (Carlton, 1999). Moreover, this rate can also be partially circumvented by using several phages in one preparation much like using two or more antibiotics simultaneously. When resistance against a given phage occurs, a new phage can be created to target and destroy the new strain. Some protocols on isolation of phage have been mentioned in literature (Garcia et al., 2009; Moineau and Fortier, 2009). The selection and screening a new phage is faster than the development of novel antibiotics which can take up to several years (Sulakvelidze et al., 2001).

CONCLUSION

The use of bacteriophages as antimicrobial agents controlling pathogenic bacteria has appeared as a promising new strategy and it seems that phage therapy may provide a good alternative solution to antibiotics. The abundance of phages in the environment highlights their potential use for control of pathogenic bacteria in food and animals. For an effective treatment, bacteriophages should be (1) present in high concentrations, (2) stable over time and in *in vivo* conditions, (3) able to meet the bacteria without any restriction, and (4) able to replicate. For that, some points should be taken into account:

- Use a high level of phage concentration
- Use phage to treat the bacterial infection as soon as possible
- Test the stability of phage in real environmental conditions
- Protect the phage by microencapsulation
- Screen and develop the cocktail of phage lytic which is able to infect many bacterial strains
- Use a polyvalent bacteriophages with broad cross-strain lytic activity or develop a phage cocktail to lyse the majority of bacteria strains and limit the development of resistance to phage

However, several challenges may arise in phage therapy such as:

- phages can be neutralized by antibodies or other components in the matrices
- bacteria may develop on several sites which are inaccessible to phage.

There is a need to develop a model which approaches the *in vivo* conditions to elucidate the factor influence on the infection capacity of phages before *in vivo* application.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 October 2013; accepted: 23 January 2014; published online: 18 February 2014.
Citation: Ly-Chatain MH (2014) The factors affecting effectiveness of treatment in phages therapy. Front. Microbiol. ***5****:51. doi: 10.3389/fmicb.2014.00051*
This article was submitted to Virology, a section of the journal Frontiers in Microbiology.