Phages of lactic acid bacteria: The role of genetics in understanding phage-host interactions and their co-evolutionary processes

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ABSTRACT

Dairy fermentations are among the oldest food processing applications, aimed at preservation and shelf-life extension through the use of lactic acid bacteria (LAB) starter cultures, in particular strains of Lactococcus lactis, Streptococcus thermophilus, Lactobacillus spp. and Leuconostoc spp. Traditionally this was performed by continuous passaging of undefined cultures from a finished fermentation to initiate the next fermentation. More recently, consumer demands on consistent and desired flavours and textures of dairy products have led to a more defined approach to such processes. Dairy (starter) companies have responded to the need to define the nature and complexity of the starter culture mixes, and dairy fermentations are now frequently based on defined starter cultures of low complexity, where each starter component imparts specific technological properties that are desirable to the product. Both mixed and defined starter culture approaches create the perfect environment for the proliferation of (bacterio)phages capable of infecting these LAB. The repeated use of the same starter cultures in a single plant, coupled to the drive towards higher and consistent production levels, increases the risk and negative impact of phage infection. In this review we will discuss recent advances in tracking the adaptation of phages to the dairy industry, the advances in understanding LAB phage-host interactions, including evolutionary and genomic aspects.

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Phage adaptation to the processing environment

Phages encountered in a food fermentation environment may persist in the production facility for long periods and often adapt to cope with technological hurdles presented to them such as pasteurisation and other thermal and chemical sanitation procedures. In the dairy industry, milk may be treated in a number of
ways prior to its use in the fermentation process. These include low temperature long time (LTLT) treatment (63 °C for 30 min), high temperature short time (HTST) treatment (72 °C for 15 s) and the yoghurt elaboration treatment (82 °C for 10 min). The thermal inactivation kinetics of eight *Lb. paracasei* phages was assessed and three of the phages were capable of survival at 90 °C for 30 min in reconstituted skimmed milk or enriched reconstituted medium, while survival in MRS broth at this temperature was significantly lower with complete inactivation at 15 min (Capra et al., 2009). This study, however, highlights the capability of the majority significantly lower with complete inactivation at 15 min (Capra et al., 2009). This study, however, highlights the capability of the majority of tested phages to survive beyond the standard phage inactivation treatment of 90 °C for 10 min. Similarly, the *Lb. delbrueckii* phage Ldb is thermally resistant surviving extended treatments at 60, 70 and 80 °C and surviving beyond 30 min at 90 °C (Wang et al., 2010).

Furthermore, phages have long been assumed to require calcium, magnesium or other divalent cations for successful infection. In an effort to reduce phage infection of starter cultures, the dairy industry employs various phage inhibitory media, which incorporate phosphate buffers and citrate salts (Gulstrom et al., 1979), for the cultivation of starter cultures prior to their addition to milk. Physical characterisation of many new phage isolates includes defining the requirement of calcium or divalent cations for adsorption or infection of their hosts. Phage Ldb requires calcium for complete lysis of its bacterial host, while adsorption of this phage to its host appears to be calcium independent (Wang et al., 2010). Differential behaviour of *Lb. paracasei* phages in response to calcium was observed with phage MLC-A8 being able to cause lysis in the absence of calcium, while adsorption of this phage to its host was shown to be more efficiently than its calcium-dependent counterparts irrespective of the presence or absence of calcium (Capra et al., 2009). Additionally, *Lb. brevis* phage SA-C12, capable of infecting beer spoilage strains, is calcium-independent in liquid and solid media analyses (Deasy et al., 2011). A study of lactococcal P335 and 936-type phages, isolated in Argentina, showed that most of these phages are fully calcium independent on calcium to effect host lysis in liquid medium, while one 936-type and a P335-type phage isolate did not require calcium for lysis (Suarez et al., 2008). In all cases calcium was not required for phage adsorption which is in line with other studies. In contrast to this, a temperate *Lb. delbrueckii* phage does exhibit a calcium-dependent phage adsorption phenotype (Trucco et al., 2011). The evolutionary pressure to compete under certain conditions may have dictated the emergence of LAB phages capable of attaching to and infecting their hosts in low mineral conditions. It is noteworthy that for phages that do not require divalent cations for adsorption or entry into the host, the lytic cycle is expedited in the presence of these cations (Briggiler Marco et al., 2009; Deasy et al., 2011).

Certain environmental conditions appear to have a greater influence on phage adsorption capabilities than the presence of divalent cations. For example, phage adsorption is influenced by pH and temperature with lactococcal 936- and P335-type phages adsorbing optimally at pH 6–7, while pH values between 5 and 7 appear to be optimal for temperate phages of *Lb. delbrueckii* and *Lb. plantarum* (Briggiler Marco et al., 2009; Suarez et al., 2008; Trucco et al., 2011). Furthermore, and not surprisingly, the majority of tested phages adsorb most efficiently at temperatures at which the host strain grows optimally. Starter strain rotation is often employed in the dairy industry to remove propagation opportunities for phages on industrially important strains (Sing and Klaenhammer, 1993). These strains are phage-unrelated and, as such, retard the proliferation of phages in the dairy plant, however, these systems have limited success as phages continually adapt and overcome these technological hurdles. In food fermentation facilities, various strategies to control phage and other microbial contaminants are performed including sanitation with chemicals such as sodium hypochlorite and peracetic acid. It is now clear that phages are also adapting to such chemical treatments, which may force the implementation of a chemical treatment rotation scheme in such facilities. For example, in one study *Lb. paracasei* phages, which were characterised with respect to resistance to processing and sanitation treatments, were shown to be resistant against high pressure homogenisation, treatments with ethanol, and exposure to sodium hypochlorite at less than 400 ppm (Mercanti et al., 2012). On the other hand, treatments with peracetic acid and alkaline chloride foam (among others) were significantly more effective with 99% phage inactivation (T90) in less than 2 min (Mercanti et al., 2012). In response to the adaptation of phages to processing treatments, there is a constant drive to develop and implement new anti-phage treatments and strategies. The recent finding that quinolinilporphyrin may be an effective agent preventing lactococcal phage proliferation is convincing evidence for such an approach (Vodzinska et al., 2011). Each individual fermentation facility implements a regime of sanitation and strain rotation to prevent and combat the phage problem. However, evidence from recent reports would suggest that these strategies should be regularly assessed for their effectiveness in relation to the phage population of that plant (Mercanti et al., 2012; Vodzinska et al., 2011).

Following successful phage adsorption and penetration, phages establish lysogeny or proceed to a lytic cycle. Phage replication, whether temperate or virulent, results in the co-evolution of the bacterial and phage populations. Co-evolution facilitates diversification by sequentially favouring innovations in lineages, which, in turn, favours innovations in the other lineage. However, novel key innovations may occur less frequently than minor unfavourable innovations to which the partner may not currently be adapted to (Thompson, 2012). Arguably the best examples of bacteria and phage interactions driving co-evolution are related to bacterial defence mechanisms and phage circuitry thereof. LAB may acquire phage defence systems through Horizontal Gene Transfer (HGT) and such systems can impede all aspects of phage replication and may interfere with phage adsorption, prevent DNA injection, degrade incoming DNA, and abort infection (Labrie et al., 2010).

**LAB genetics and BIM developments**

Developments in genetic tools and molecular sequencing technologies have greatly facilitated our understanding of phage-host interactions. There is a rapid emergence of sequencing data available for all LAB strains. At present, Genomes OnLine Database (GOLD www.genomesonline.org) reports that there are: 11 fully sequenced lactococcal genomes, 5 *S. thermophilus* genomes, 110 lactobacilli genomes, 10 *Leuconostoc* genomes and 4 pediococci genomes. However, not all of these genomes have been published. The number of fully sequenced LAB phage genomes has increased even more dramatically in the last number of years (Table 1). Just recently, 28 new 936 phage genomes were sequenced and compared (Castro-Nallar et al., 2012). LAB phage genetics has been dominated by phages infecting lactococci and this is reflected by the number of sequenced genomes of phages infecting this host (Table 1). Additionally, the availability of genome sequences of rarely isolated or emerging lactococcal phages is very useful in assessing the role of phage in host evolution and genetics (Deveau et al., 2006). By sequencing industrially significant bacteria, genetically related strains with similar technological properties may be identified with resistance to the current phage population(s). In parallel, sequencing emerging phage populations may allow the identification of weaknesses or imminent problems in starter strains. This is expected to facilitate
the rational design of anti-phage mechanisms to prevent phage initiation of infection and subsequent replication.

Many important technological phenotypic traits are strain-specific and therefore replacing a phage-sensitive strain with a phage-insensitive strain when a phage emerges is often not possible or desirable (Capra et al., 2011). To overcome this problem, Bacteriophage Insensitive Mutants (BIMs) of the original strain may be isolated which are resistant to the offending phage while retaining their technologically important attributes.

There are many ways in which a strain can become resistant to a phage, for example by acquiring plasmid-borne natural phage-resistance mechanisms (specifying for example restriction/modification systems and/or abortive infection proteins) (Labrie et al., 2010; O’Driscoll et al., 2004), through the expansion of CRISPR/cas loci (Horvath and Barrangou, 2010), or by spontaneous mutations in the phage-host receptor or other host-dependent factors that are required for phage proliferation following DNA adsorption (Garneau and Moineau, 2011).

Barrangou et al. (2007) clearly illustrated the adaptation of S. thermophilus to virulent phage via the acquisition of new spacers at the leader end of the CRISPR locus. This knowledge on CRISPR/Cas systems and phage resistance has allowed the rational design of many BIM derivatives of LAB strains for industrial application. By repeatedly infecting CRISPR-containing S. thermophilus with lytic phages, it was possible to select for BIMs which acquired the necessary spacer sequences to provide a phage-resistant phenotype (Horvath et al., 2008). However, not all LAB species possess CRISPR elements (notably all sequenced L. lactis strains to date (Horvath et al., 2009)). Recently it was shown that the S. thermophilus sthe3 CRISPR locus can (artificially) be transferred to E. coli and retain its ability to provide interference against plasmids and phages. The engineered plasmid could successfully interfere with plasmid transformation by more than five orders of magnitude and lambda infection by three orders of magnitude. The authors noted how this potentially allows for the development of phage-resistant strains, and strains that are less likely to acquire and disseminate undesirable plasmids (Sapranuska et al., 2011). In dairy lacticoccal strains, however, many desirable technological properties (including phage defence mechanisms) are plasmid-encoded and this may be an underlying reason why lactococcal (starter) strains do not possess such systems.

There are many published methods for spontaneous BIM generation in various LAB. They have shown varying degrees of success and are often dependent on the species and strains used (Capra et al., 2011). Generally, they involve the challenge of cultures with high levels of single (Coffey et al., 1998; Guglielmiotti et al., 2007) or multiple (Marco et al., 2011) bacteriophages. Mills and colleagues 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 (Mills et al., 2007). As selection towards fast growth is applied during the fermentation process and since BIMs will frequently exhibit a reduced growth phenotype, many BIMs tend to revert back to fast growth and bacteriophage sensitivity (Marco et al., 2011; Mills et al., 2007). Although the molecular mechanisms have not been fully elucidated, it is thought that certain cell surface receptors for (some) LAB phages, such as teichoic acids or CWP (Table 2), are associated with many essential functions (Neuhaus and Baddiley, 2003). Changes in their structure or mutations in key biosynthetic genes are expected to cause detrimental effects on growth and/or cell division, as demonstrated by the formation of abnormally long-chained lactococci carrying mutations in cell wall polysaccharide (CWP) synthesis genes (Chapot-Chartier et al., 2010; Dupont et al., 2004). This may thus lead to selective pressure to revert back to a phage-sensitive phenotype. Therefore phenotypic stability of BIMs is an important factor to consider, though this may vary amongst BIMs of different

### Table 1

<table>
<thead>
<tr>
<th>Host bacterium</th>
<th>No. of sequenced genomes at family level</th>
<th>No. of sequenced genomes; classification, where relevant</th>
<th>Genome size range (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactococcus lactis</td>
<td>57 Siphoviridae</td>
<td>40; 936 species</td>
<td>27.5–32.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7; P335 species</td>
<td>32.3–40.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2; c2 species</td>
<td>22.2–22.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1; 949 species</td>
<td>114.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1; 1708 species</td>
<td>55.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1; Q54 species</td>
<td>26.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1; P087 species</td>
<td>60.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1; 1358 species</td>
<td>60.1</td>
</tr>
<tr>
<td>2 Pseudomonadae</td>
<td>1; K34 species</td>
<td>79.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1; P034 species</td>
<td>18.8</td>
</tr>
<tr>
<td>S. thermophilus</td>
<td>11 Siphoviridae</td>
<td>5; pac-type</td>
<td>34.7–43.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5; cos-type</td>
<td>34.8–40.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1; Unclassified</td>
<td>37.2</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>20 Siphoviridae</td>
<td>2; Lc-No species</td>
<td>36.5–39.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18; Unclassified</td>
<td>31.1–49.4</td>
</tr>
<tr>
<td>2 Myoviridae</td>
<td>1; SP01-like</td>
<td>131.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1; Unclassified</td>
<td>142.1</td>
</tr>
<tr>
<td>Leuconostoc</td>
<td>1 Siphoviridae</td>
<td>1; Unclassified</td>
<td>29.5</td>
</tr>
<tr>
<td></td>
<td>1 Unknown</td>
<td>1; Unclassified</td>
<td>2.4</td>
</tr>
<tr>
<td>Pedicoccus</td>
<td>1 Siphoviridae</td>
<td>1; Unclassified</td>
<td>38.0</td>
</tr>
</tbody>
</table>

Note: cos-type phages are those with cohesive ends and the cos site is a recognition port for the terminase protein to terminate DNA packaging by cleaving the DNA at unique cosN sequences at either end of the genome while pac-type phages may terminate packaging by cleaving firstly at a pac site and subsequently at a random site once the capsid is full. This is also termed “headful packaging”.

### Table 2

<table>
<thead>
<tr>
<th>Primary host species</th>
<th>Phage (species, where relevant)</th>
<th>Receptor or proposed receptor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactococcus lactis ssp. cremoris</td>
<td>sk1 (936)</td>
<td>*CWP</td>
<td>(Chapot-Charter et al., 2010; Dupont et al., 2004)</td>
</tr>
<tr>
<td>Lactococcus lactis ssp. lactis</td>
<td>TP901-1 (P335)</td>
<td>Unknown carbohydrate</td>
<td>(Spinelli et al., 2006)</td>
</tr>
<tr>
<td>Lactobacillus delbrueckii</td>
<td>c2 (C2)</td>
<td>Phage infection protein</td>
<td>(Geller et al., 1993)</td>
</tr>
<tr>
<td>Lactobacillus casei</td>
<td>LL-H</td>
<td>Lipo-choi acid</td>
<td>(Raisanen et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>JCL1032</td>
<td>Unknown carbohydrate</td>
<td>(Ravin et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>YAB</td>
<td>*CWP</td>
<td>(Quiberoni et al., 2004)</td>
</tr>
<tr>
<td>Lactobacillus plantarum</td>
<td>J-1</td>
<td>*CWP</td>
<td>(Yokokura, 1977)</td>
</tr>
<tr>
<td></td>
<td>Phi2</td>
<td>*CWP</td>
<td>(Douglas and Wolin, 1971)</td>
</tr>
<tr>
<td>Lactobacillus helveticus</td>
<td>CNRZ 822B1</td>
<td>Teichoic Acid</td>
<td>(Douglas and Wolin, 1971)</td>
</tr>
<tr>
<td>Streptococcus thermophilus</td>
<td>CV3</td>
<td>s-layer protein</td>
<td>(Ventura et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>5093</td>
<td>*CWP</td>
<td>(Quiberoni et al., 2004)</td>
</tr>
<tr>
<td>Leuconostoc mesenteroides</td>
<td>Phi1-A4</td>
<td>Unknown carbohydrate</td>
<td>(Mills et al., 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unknown carbohydrate</td>
<td>(Lu et al., 2010)</td>
</tr>
</tbody>
</table>
LAB species/strains and is likely dependent on several host and environmental factors. High stability of BIMs of some lactobacilli and *S. thermophilus* strains is likely not due to mutations in host cell receptors, but by the expansion of CRISPR loci, which has no other observable negative effect on the cell (Horvath and Barrangou, 2010). Probably due to the lack of CRISPR loci, the generation of BIMs for *L. lactis* often results in negative growth phenotypes with a concomitantly high frequency of BIM reversion; consequently stable and technologically exploitable BIMs are difficult to obtain for this species.

Inevitably, bacteriophages will evolve to overcome any conformational changes in the host receptor. Analysis of bacteriophage 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 (Duplessis et al., 2006; Ravin et al., 2002; Uchiyama et al., 2011). Such information highlights the importance of sequencing strategies in order to understand the evolutionary and molecular processes that allow phages to overcome host barriers. Furthermore, this knowledge provides a platform for the development of the next generation of phage-resistant starter cultures against related groups of phages rather than individual parasites in the dairy industry.

**Host-encoded receptors for LAB phages**

The outer surface of Gram positive bacteria consists of a thick layer of peptidoglycan decorated with various proteins and (poly)saccharides (Delcour et al., 1999). The majority of studied bacteriophages infecting Gram positive bacteria are thought to recognise their host in a two-step manner. First, a reversible interaction occurs via a saccharide moiety on the cell surface leading to a second, irreversible interaction, which can either be with the same receptor or different surface component (Baptista et al., 2008; Geller et al., 1993). This reversible-irreversible interaction is proposed to allow the bacteriophage to diffuse laterally across the cell surface upon initial reversible adsorption, increasing the probability of encountering the final and perhaps host-specific receptor.

For Gram positive bacteria, wall teichoic acids (WTA) (Baptista et al., 2008), lipoteichoic acids (LTA) (Raisanen et al., 2004), and cell wall polysaccharides (CWP) (Shibata et al., 2009) have all been associated with bacteriophage adsorption (Table 2). Most LAB phages display a very narrow individual host range (Guglielmotti et al., 2009; Rousseau and Moineau, 2009) and phage sensitivity has been linked to strain-specific decoration or side chain formation of cell surface polysaccharides (Rai et al., 2007). Extracellular polysaccharides (EPS) are not covalently attached to the peptidoglycan and may thus be loosely associated with the cell wall. EPS production is usually strain-dependent as EPS genes are often associated with mobile elements. EPS may aid or hinder adsorption (Forde et al., 1999; Rodriguez et al., 2008), but are generally not considered to be bacteriophage receptors (Deveau et al., 2002) and therefore will not be discussed further in this review. The receptors of LAB phages have been the focus of many recent studies with those of *Lactococcus*, *Streptococcus* and *Lactobacillus* dominating, presumably due to their industrial relevance.

**Lactococcus lactis**

Of the three major phage species infecting *L. lactis*, only the receptor for the c2-type species has been definitively identified (Geller et al., 1993; Monteville et al., 1994). It follows the conventional reversible saccharide binding prior to irreversible binding of the membrane protein termed Pip (phage infection protein). In contrast, the precise molecular nature of receptors for lactococcal phages of the 936 and P335 species have yet to be determined. Studies relating to 936 phage-host interactions have indicated that the cell wall components, and not membrane or proteinaceous elements, inhibited bacteriophage adsorption (Geller et al., 2005; Valyasevi et al., 1994).

Breum and colleagues proposed that different P335 phages perhaps use the same receptor but have different routes for DNA delivery (Breum et al., 2007). *L. lactis* 3107 mutants were generated by chemical (EMS) mutagenesis that were resistant to infection by P335-type species (TP901-1 and *φ*L3C), although they were still sensitive to *c2-* and 936-type phages. Of the mutants generated, two were insensitive to adsorption by either of the two P335-type phages, while three mutants were impervious to TP901-1, yet sensitive to φL3C. Further experiments showed that TP901-1 was capable of binding to the mutant 3107 isolates and electron micrographic analysis determined DNA ejection was inhibited. This led the authors to suggest that different receptors are used for TP901-1 and LC3, or that different routes of DNA injection are employed by these two phages.

Studies on purified receptor binding proteins (RBPs) of the lactococcal phages p2 and TP901-1 (936- and P335-type species, respectively) revealed that these proteins had a high affinity for glycerol among other carbohydrates (Spinelli et al., 2006). Further electron microscopy (EM) studies of entire baseplates and the respective components of phages TP901-1 and Tuc2009 demonstrated that the TP901-1 base plate contains fifty four copies of the receptor binding protein (RBP), thus leading to an equivalent number of possible interaction points with a presumed saccharide moiety on the host cell surface (Bebecua et al., 2010; Sciara et al., 2008). In contrast, phage p2 contains six trimers of the RBP, and consequently presenting eighteen possibilities for saccharide binding (Sciara et al., 2010). 

Chapot-Chartier and colleagues reported the structure of a novel CWP present in *L. lactis* MG1363 (Chapot-Chartier et al., 2010). Mutants deficient in this polysaccharide became resistant to the 936-type species phage sk1. Previously, the operon encoding the suspected biosynthetic machinery for an analogous polysaccharide in *L. lactis* IL1403 was linked to 936-type phage infection (Dupont et al., 2004). Current evidence, while suggestive of a direct role, is inconclusive as to whether this CWP is indeed the cell surface receptor for 936 species phages as mutants lacking this CWP have growth defects and display a long-chain phenotype. These growth defects could therefore impede phage infection by other means.

**Streptococcus thermophilus**

Phage host receptors are currently unknown for streptococcal phages (Quiberoni et al., 2010), however, inhibition and adsorption studies have determined that streptococcal phage-host receptors are most likely carbohydrate in nature as the cell envelope, and not membrane or proteinaceous elements, are able to adsorb bacteriophages (Quiberoni et al., 2000). The ability of such cell envelope preparations to adsorb bacteriophages was reduced after enzymatic treatment with mutanolysin, suggesting that the receptor is part of the peptidoglycan or a covalently linked polymer (Binetti et al., 2002). Interestingly and similar to lactococcal phage studies, carbohydrates present in the CWP, specifically N-acetylgalactosamine, rhamnose and glucosamine, were also shown to inhibit streptococcal phage (Binetti et al., 2002). CWP receptors in *S. thermophilus* have not been studied in detail, however, CWP in the related pathogenic *Streptococcus pneumoniae* have been thoroughly characterised and have been shown to have the potential for immense variety due to different glycan composition and glycosidic linkages (Yother, 2011).
Similar to lactococcal phages, individual S. thermophilus phages have a very narrow host range (Cuglievomotti et al., 2009). However, in contrast to their lactococcal counterparts they are genetically and morphologically less diverse, consisting of a large polythetic group divided into two groups based on their DNA packaging mechanism (Desiere et al., 1998; Le Marrec et al., 1997; Quiberoni et al., 2010). Host specificity has been studied in detail in Streptococcus and variable regions within the receptor binding protein (RBP) are thought to be the cause of host specificity (Duplessis and Moineau, 2001). A direct correlation between the VR2 sequence of the RBP and host range appears to exist (Zinno et al., 2010). However, as with phages infecting other species, host contributing factors, such as CRISPR/CAS could also play a role. Recently, a novel streptococcal phage was isolated that did not appear to encode an anti-receptor gene, suggesting that this phage interacts with its host in a different manner, uses a completely different receptor or contains an RBP whose structure is conserved while its amino acid sequence is divergent (Mills et al., 2011).

### Lactobacillus spp

The only characterised phage-host receptor for a Lactobacillus-infesting phage is that of the small isometric-headed Lb. delbrueckii phage LL-H. LTA isolated from the host completely inhibited phage infection when present in picogram quantities, whereas LTA from other strains inhibited the phage up to 100 times less efficiently (Raisanen et al., 2007; Raisanen et al., 2004). Structural characterisation of LTAs of host cells and BIMs showed variation in the degree of α-glucosyl and D-alamyl substitutions of the LTA backbone. Molecular characterisation suggested that as the number of substitutions increases, phages are increasingly less efficient in adsorbing to a potential host. Further investigation with bacteriophages from various homology groups demonstrated that only phages from groups a and c were able to utilise LTA as cell surface receptors, implying that Lb. delbrueckii phages from different homology groups probably employ more than one cell surface receptor (Raisanen et al., 2007). Quiberoni and colleagues investigated Lb. delbrueckii phage from homology group b and found that these phages adsorb in an irreversible manner to cell walls of sensitive hosts and not to membrane or proteinaceous components (Quiberoni et al., 2004). Furthermore, the prolate-headed phage JCL1032, which is unrelated to LL-H, was shown to infect the same host strain and is likely to employ an alternative cell surface receptor, as BIMs unable to adsorb LL-H were successfully infected by JCL1032 (Ravin et al., 2002). For Lb. helveticus phage CNRZ 832-B1, the S-layer proteins of several strains was found to be essential for phage adsorption (Ventura et al., 1999).

Bacteriophages infecting other industrially important LAB, such as Weisella, Oenococcus, Pediococcus and Leuconostoc species are considerably less well investigated in comparison to the LAB species previously discussed. As such, virtually no information is available on how bacteriophages from these species interact with their host, while only a small number of bacteriophage genomes infecting these species are available. The predicted anti-receptor gene of Leuconostoc mesenteroides phage Ψ1-A4 (Lu et al., 2010) shows homology with L. lactis phage QS4 (Fortier et al., 2006), which has a L. lactis c2-type phage-like RBP. While research into the genetics of these LAB phages is in its infancy, the rate of technological advances in recent years together with renewed interest in phages of these genera through ecological studies is expected to bring a torrent of information in the coming years that may rival that of the more dominant LAB genera. Such data will permit an advanced understanding of the evolution and diversity of phages of the LAB. In concert with this, there will be a greater understanding of host diversification in response to phage infection and the role of phages in host adaptive responses.

### Host response to infection: transcriptomics and other new technologies

Gram negative models of phage-host interaction in Escherichia coli with bacteriophage lambda and T7 infecting the same strain show striking differences in host response. Fifty seven non-essential host genes, involved in central metabolism and regulation of the lambda receptor, have been identified to be required for efficient phage lambda replication (Maynard et al., 2010), in stark contrast to bacteriophage T7 in which only eleven such genes were identified (Qimron et al., 2006). This demonstrates that host response to different phages infecting the same species can be drastically different, and in some cases, e.g. bacteriophage lambda, demonstrate a highly integrated molecular network (Maynard et al., 2010).

While much information is available on the host response of Gram negative bacteria to their host, very little is known about the response of Gram positive bacteria to phage infection, especially LAB. However, some recent studies in Lactococcus, Lactobacillus and Streptococcus reveal host mechanisms activated in response to phage infection. Thus far, no sequenced genomes of LAB phages have been found to contain genes encoding DNase, although two instances of phages infecting L. lactis with DNase activity have been reported (Michelsen et al., 2007; Powell et al., 1992). This suggests that host chromosomes are still intact during infection and a host may still be physiologically active. Also, it has been demonstrated that S. thermophilus phages can adsorb to cells at 0 °C, but cannot proceed with DNA ejection, suggesting that an actively metabolising bacterium is required for DNA ejection for some S. thermophilus phages (Binetti et al., 2002).

The transcriptional response of L. lactis IL1403 to bacteriophage c2 during the early stage of infection using whole genome DNA microarrays was recently assessed (Fallico et al., 2011). During early infection, cells appeared to be metabolically active and capable of directing infection in a proposed four strand approach including energy conservation, maintenance of the proton motive force, alanulation of cell walls and induction of membrane stress proteins. Of particular interest is the up-regulation of the dltABCD operon, which is responsible for the incorporation of D-alamyl esters onto peptidoglycan and LTA (Neuhaus and Baddiley, 2003). LTA, as mentioned earlier, are candidate phage cell surface receptors for L. lactis. It has been suggested that the up-regulation of the dltABCD operon acts as a steric receptor blocking mechanism reminiscent of superinfection exclusion systems, preventing further phage infection (Neuhaus and Baddiley, 2003). Furthermore, it may prevent progeny phages from attaching to cell envelope debris of lysed cells if the receptor is not present in the correct configuration. Global regulator SpxB and the two-component system CesSR, which are involved in the adaptive response of L. lactis to various environmental stresses, were also identified in this study as being differentially transcribed, suggesting that they are part of a strategy aimed at protecting cells against envelope stress.

Host response to phage infection in S. thermophilus LMD-9 by the lytic phage DT1 was monitored over a forty minute time course during which a transient increase in CRISPR gene expression 5 min post-infection was observed followed by a decrease in overall gene expression (Goh et al., 2011). It has been suggested that this is likely evidence of integration of novel spacers when faced with phage infection. Interestingly, one of the first applications of microarray technology with respect to phages of Gram positive bacteria was the definition of the transcriptional profiles of the cos and pac phages DT1 and 2972, respectively (Duplessis et al., 2005).

In recent years, microscopy and flow cytometry methods have been utilised to monitor LAB host-phage interactions. Flow cytometry
monitoring of L. lactis infected with 936-, c2- or P335-type species all showed the same characteristic defects in growth during early infection, where the long chain phenotype of actively growing L. lactis ceases and single cells appear suggesting arrest in cell growth but continuation of division (Michelsen et al., 2007). Additionally, cell walls were shown to be partially degraded during infection with all phage species, suggesting that intracellular replication impacts on cell wall metabolism. Host-phage interactions in Lb. helveticus were studied using atomic force microscopy and changes in cell morphology were visible during early infection, with a protuberance at the distal part of the cell (Zago et al., 2012). The interaction between phage and host has remained one of the most dominant aspects of phage biology in recent years as this is believed to be essential for the development of effective anti-phage strategies in the dairy industry, as well as for the design of a phage and host classification system based on (anti)receptor type that may benefit phage therapy strategies.

**LAB cell surface: the interface**

As the cell surface is the first point of contact between bacteriophage and its host, any advances in the nature of the cell surface may shed light on their interaction, as well other important functional roles. In recent years there have been many interesting developments in understanding the composition and spatial organisation of LAB cell surfaces. The exocellular biology of lactobacilli is particularly well characterised (for an extensive review, see Kleerebezem et al., 2010) due to their extensive use as probiotics. Physicochemical analysis has revealed striking diversity in cell surface properties of different LAB. Differences in these properties can affect binding of cells to surfaces and food matrices (Ly-Chatain et al., 2010). Fifty L. lactis strains were examined for their surface properties and groups of strains with highly hydrophobic cell surfaces and strains with unusually low charged cell surfaces were identified (Giaouris et al., 2009). Furthermore, the diversity of physicochemical properties could not be linked to the origin or subspecies of the strain. It has also been demonstrated that LTA composition is determined by the available carbon source, with cells of L. lactis IL1403 grown on galactose having LTA containing less galactose compared to cells grown on glucose, although the effect on phage proliferation is unclear (Steen et al., 2008).

Several studies have recently utilised atomic force microscopy (AFM) to observe the spatial organisation of peptidoglycan, individual CWP and teichoic acids on the cell surface of live LAB (See for a review Tripathi et al., 2012). L. lactis cells display a smooth cell surface, whereas mutants lacking CWP show periodic bands of peptidoglycan running parallel to the short axis of the cell (Andre et al., 2010; Chapot-Chartier et al., 2010). This study not only demonstrated the nature of peptidoglycan in L. lactis, but additionally that CWP linked to infection of 936-type phages is homogeneously distributed across the cell surface. Furthermore, it was determined that distribution of WTA in Lb. plantarum is heterogeneous, while the cell poles are devoid of WTA leading to highly polarised surface morphology, indicating that this plays a key role in cell morphogenesis (Andre et al., 2011). AFM also revealed the co-existence of CWP of different nature on the cell surface of L. rhamnosus GG (Francius et al., 2008). Complementing this, a lectin microarray has been developed to compare cell surface “glycomes” of 16 Lb. casei strains. Results demonstrated that lectin binding profiles are strain unique, further highlighting the massive inter-strain variability in cell surface polysaccharides (Yasuda et al., 2011).

With the abundance of completed genomes available, there have been a few attempts to perform comparative analyses of “exoproteomes” of LAB in recent years (Kleerebezem et al., 2010; Zhou et al., 2010). Kleerebezem et al. (2010) extracted 2451 putative proteins from 13 Lactobacillus genomes and found large disparity in predicted exoproteomes between species. A genome-scale comparative analysis of the predicted exoproteomes from all LAB genomes available was performed and universal families of proteins were identified which are predicted to be essential for LAB, as well as species and even niche-specific protein families (Zhou et al., 2010). The results were formatted into an LAB-secretome database and will undoubtedly be valuable for probing phage-host interactions in the future. The grand scale of the exoproteomes in LAB means there is likely to be huge diversity in any cell surface LAB phage-host protein receptor. Phage-host interaction analyses are aided by the consistent isolation of new phage isolates that can be studied and compared to older isolates to assess genotypic and phenotypic trends.

**Conclusions and future perspectives**

It is evident from recent publications on LAB phages that a two-pronged approach is required in order to fully understand phage biology. This infers that an understanding of both phage and host genetics and biology is essential in order to define phage requirements for infection and to determine how phage and host co-evolve to counteract and adapt to the threat posed by the other. The problem of phage infection of LAB used in various food fermentations will never be eliminated; however, the fundamental approach which has been applied to gain insights into the interactions of the phage and host is certainly an invaluable contribution towards developing tools and strategies to control and curb the problem of phage infection during food fermentations. The development of transcriptomic tools to delineate the effect of phage infection on the host gene expression pattern is possibly one of the more significant developments in the area of phage biology. The relative ease with which this technology may now be applied has several implications for the fundamental analysis of phage-host interactions as well as industrial applications. As this technology grows and such studies emerge, the pattern of phage-host interactions and the requirements for phage infection, increasingly intricate control measures may be developed and implemented. Additionally, the surge in the availability of phage and host genome sequences provides a wealth of information and as further combinations of phage and host bacterial genomes are sequenced, the relationship between phages and their hosts will become increasingly clear. Furthermore, it may aid to identify of proteins and carbohydrates that phages require as primary and secondary receptors, which information may be used to define genetic markers to screen industrial strain collections for phage-unrelated strains. The future of LAB phage biology point toward “-omics” as well as structural approaches to identify and analyse the molecular players that facilitate phage-host interactions. These players and their mechanisms of action will allow the development of new control measures, which should be implemented in conjunction with natural host-encoded phage-resistance systems and perhaps novel and rotating plant sanitisation schemes.

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**References**


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