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Biodiversity of bacteriophages infecting *Lactococcus lactis* starter cultures

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ABSTRACT

In the current study, we characterized 137 *Lactococcus lactis* bacteriophages that had been isolated between 1997 and 2012 from whey samples obtained from industrial facilities located in 16 countries. Multiplex PCR grouping of these 137 phage isolates revealed that the majority (61.31%) belonged to the 936 group, with the remainder belonging to the P335 and c2 groups (23.36 and 15.33%, respectively). Restriction profile analysis of phage genomic DNA indicated a high degree of genetic diversity within this phage collection. Furthermore, based on a host-range survey of the phage collection using 113 dairy starter strains, we showed that the c2-group isolates exhibited a broader host range than isolates of the 936 and P335 groups.
Key words: lactic acid bacteria, lactococcal phage group, whey sample, dairy starter strains

INTRODUCTION

Bacteriophages or phages (i.e., viruses that infect bacteria) are the most abundant and diverse biological entities present in the earth's biosphere (Garneau and Moineau, 2011; Marco et al., 2012). Ecological niches such as dairy fermentation plants constitute an ideal environment for the propagation and persistence of phages infecting bacterial starter strains, where such infections may cause fermentation issues of varying severity (Marco et al., 2012). Bacterial growth to high cell densities of specific lactic acid bacteria, such as *Lactococcus lactis*, *Streptococcus thermophilus*, *Leuconostoc* spp., and *Lactobacillu*s spp., is required for a successful dairy fermentation, but it also provides a perfect "breeding ground" for phages that are present in the manufacturing environment (Garneau and Moineau, 2011). Characterized phages infecting *Lc. lactis* possess a double-stranded DNA genome and belong to the *Caudovirales* order, and are members of either the long-tailed *Siphoviridae* or the short-tailed *Podoviridae* families (Deveau et al., 2006; Mahony and van Sinderen, 2014). Among the 10 currently recognized lactococcal phage groups, members of the so-called 936, P335, and c2 groups appear to be the most frequently encountered phages in the dairy environment (Deveau et al., 2006). Isolation and enumeration of phages from milk and whey samples is typically performed by plaque assays (Lillehaug, 1997). In recent years, molecular and diagnostic tools have become more commonly used for rapid and reliable detection of these 3 major groups, primarily by means of a multiplex PCR approach (Labrie and Moineau, 2000; del Rio et al., 2007). Several studies have shown that 936-group phages are the most endemic and problematic group of lactococcal phages in fermentation facilities (Kleppen et al., 2011; Mahony et al., 2012; Murphy et al., 2016). Thus, members of this group have been extensively characterized (phenotypically and genetically) so as to improve our understanding of their morphology, interactions with the host, genetic diversity, and sensitivity to biocidal and thermal treatments (Crutz-Le Coq et al., 2002; Dupont et al., 2004; Mahony et al., 2006, 2013; Murphy et al., 2014, 2016). In contrast to the well-studied 936 phages, public databases currently hold just 10 complete c2-group genome sequences, which typically exhibit extensive DNA homology (Schouler et al., 1994; Lubbers et al., 1995; Millen and Romero, 2016). Genetic diversity within this group is largely confined to 3 open reading frames (ORF), whose encoded products are believed to be responsible for the observed host-range differences (Stuer-Lauridsen et al., 2003; Millen and Romero, 2016). Comparative genomic analysis of phages belonging to the P335 group has revealed significant genetic diversity and the absence of a "core" genome; that is, no single gene is conserved among all assigned members of this phage group (Mahony et al., 2014). The ge-

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nomes of members of the P335 group exhibit extensive mosaicism as a presumed consequence of recombination between related phages that may involve virulent and temperate members of this group (Labrie et al., 2008). Despite the implementation of various strategies to control (lactococcal) phages in dairy facilities (such as sanitation, culture rotations, and factory/equipment design), their presence and persistence remain a serious biotechnological problem (Marco et al., 2012). Identified phages are typically propagated on a suitable *Lc. lactis* host and then characterized using several approaches, including determination of host range, restriction enzyme profiling of phage genomic DNA, and morphological analysis by electron microscopy (Deveau et al., 2006; Szczepanska et al., 2007; Labrie et al., 2008; Mahony et al., 2013; Murphy et al., 2013). Additionally, to identify the host receptor used by certain phages and to enable the development of predictive tools and improved strain rotation strategies, lactococcal strains have been grouped based on the operon that specifies the biosynthetic machinery for the cell wall polysaccharide (**CWPS**) produced by the lactococcal host (Dupont et al., 2004; Spinelli et al., 2006; Chapot-Chartier et al., 2010; Mahony et al., 2013, 2017; Ainsworth et al., 2014a; Farenc et al., 2014; Murphy et al., 2016). To date, 3 CWPS types have been distinguished (designated types A, B, and C; Mahony et al., 2013), and additional, as-yet uncharacterized, CWPS types are known to exist (collectively designated as the unknown or U type; Ainsworth et al., 2014a).

Here, we report on the isolation and preliminary characterization of 137 lactococcal phages that were obtained from industrial facilities located in various geographical areas. Individual phages were classified as belonging to the 936, c2, or P335 phage groups, followed by host-range determination and host CWPS type identification.

MATERIALS AND METHODS

Bacterial Strains

One hundred thirteen industrial *Lc. lactis* strains were grown overnight at 30°C in 10% reconstituted skim milk and passaged in M17 broth (Oxoid Ltd., Basingstoke, UK) supplemented with 0.5% glucose (**GM17**) or lactose (LM17), where relevant. To obtain a pure bacterial culture, a fresh streak from a lactococcal overnight culture was performed on M17 agar (Oxoid Ltd.) supplemented with 0.5% glucose (GM17) or lactose (LM17), and a single colony was taken for further growth in M17 broth (using conditions as described above) and stocked in 20% sterile glycerol (Sigma Chemical, St. Louis, MO) at −20°C.

Bacteriophage Isolation

Plaque assays were performed to screen whey samples that had been collected from dairy fermentation plants located in different countries over a period of 19 yr. In this manner, 137 lytic phages were isolated (Supplemental Tables S1, S2, and S3; https://doi.org/10.3168/jds.2017-13403) and confirmed using a previously described double agar layer method (Lillehaug, 1997). A single plaque of a particular phage was propagated on an appropriate *Lc. lactis* host. Briefly, *Lc. lactis* hosts were grown to an optical density (OD_{600nm}) of approximately 0.15 to 0.2 in 10 mL of GM17 broth supplemented with 10 m*M* $CaCl_2$ (final concentration), followed by the addition of an individual plaque (using a 10-μL inoculation loop; Sarstedt, Nümbrecht, Germany), and incubated at 30°C until lysis had occurred. The lysates were then filtered through a 0.45-μm filter (Sarstedt) to remove any residual bacterial debris and stored at 4°C. Using a previously described method (Lillehaug, 1997), plaque assays were performed to determine the phage titer for each isolated phage [typically 10^8 to 10^9 plaque-forming units (**pfu**)/mL], and phage stocks were maintained as lysates at 4°C.

Phage DNA Extraction and Restriction Profile Analysis

Fifty milliliters of GM17 broth was inoculated with a (1%) fresh overnight *Lc. lactis* culture and supplemented with 10 m*M* $CaCl_2$ (final concentration). The *Lc. lactis* culture was grown until early exponential phase, and 500 μL of phage lysate (with a titer of ~10^8 pfu/mL) was added, followed by incubation at 30°C until lysis occurred. The resulting phage lysate was filtered using a 0.45-μm filter and treated with DNase and RNase to remove residual host chromosomal DNA and RNA, followed by incubation at 37°C for 40 min. Polyethylene glycol (PEG_{8000}, Sigma-Aldrich, St. Louis, MO) was added to a final concentration of 10% followed by incubation at 4°C for 16 h. Phage DNA extraction was performed as described previously (Mahony et al., 2013), and enzymatic restriction was performed by digesting phage DNA using *Eco*RV and *Eco*RI FastDigest enzymes, as recommended by the manufacturer (Thermo Fisher Scientific, Waltham, MA). The reaction mixture was incubated at 37°C for 15 min and the resulting restriction products were applied to a 1% agarose gel and visualized by using a UV transilluminator.