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Investigation of the bacteriophage community in induced lysates of undefined mesophilic mixed-strain DL-cultures using classical and metagenomic approaches

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ABSTRACT

To investigate the notion that starter cultures can be a reservoir of bacteriophages (phages) in the dairy environment, strains of three DL-starters (undefined mesophilic mixed-strain starters containing *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* and *Leuconostoc* species) were selected and induced by mitomycin C, and the whole starters were induced spontaneously as well as by mitomycin C. Frequency of induction of 17%, 26% and 12% was estimated among the isolates of the three starters, with majority of the induced phages mostly showing morphological similarity to known P335 phages, and with a fraction of them showing atypical features. Sequences of P335 quasi-species phages were found to be the most frequent entities in almost all metaviromes derived from the induced lysates. However, sequences of *Sk1virus* phages (previously 936 phages) were emerged as the predominant entities following spontaneous induction of one of the starters, suggesting a phage-carrier state. Sequences of other phages such as 949, 1706, *C2virus* (previously c2 phages) and *Leuconostoc* species could also be observed but with a lower relative frequency. Taken together, the majority of the P335 quasi-species phages could represent the induced viral community of the starters and the remaining phage groups mainly represent the background ambient viral community.

1. Introduction

Modern industrial cheese production involves the use of defined and undefined starters (Lodics and Steenson, 1990; Smid et al., 2014). Undefined starters used in mesophilic cheese productions have a relatively simple community structure with three co-existing lactic acid bacteria (LAB) species: *Lactococcus* (*Lc.*) *lactis*, and *Leuconostoc* (*Le.*) *mesenteroides* and *Le. pseudomesenteroides* (Smid et al., 2014). Undefined mesophilic starters are estimated to have > 50 different strains of *Lc. lactis* and *Le. mesenteroides/pseudomesenteroides* (Stadhouders and de Vos, 1991), with *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* and *Leuconostoc* species providing the cheese flavors, hence the name DL for *diacetylactis* (D) and *Leuconostoc* (L).

Strains of *Lc. lactis* constitute the vast majority of such starters, while strains of the two *Leuconostoc* species represent the minority. Erkus et al., by a high resolution restriction fragment length polymorphism genetic fingerprinting method, characterized eight different bacterial genetic lineages in the undefined starter Ur - a starter used in the Netherlands for a long time for the production of Gouda cheese (Erkus et al., 2013). With the exception of a single *Leuconostoc mesenteroides* lineage, all belonged to *Lc. lactis* [five subsp. *cremoris* and two subsp. *lactis* lineages].

The functionality of DL-starters during cheese manufacturing is correlated to the specific survival pattern of strains thereof (Erkus et al., 2013). The leading disrupters of such survival pattern are bacteriophages (phages), thereby influencing the amount, quality and

Abbreviations: DL-starters, starters that contain strains of *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* and *Leuconostoc mesenteroides/pseudomesenteroides* as aroma-producing bacteria; mC, mitomycin C; mC-induced, mitomycin C-induced; OD, optical density; TEM, transmission electron microscope/microscopy

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consistency of the final product.

Phage problems of dairy fermentation processes are mainly driven by members of three groups of phages attacking *Lc. lactis* - the isometric-headed *Sk1virus* (previously 936 phages) and P335, and the prolate-headed *C2virus* (previously c2 phages) group of phages (Bissonnette et al., 2000; Casey et al., 1993; Deveau et al., 2006; Jarvis et al., 1991; Moineau et al., 1996). The *Sk1virus* and *C2virus* phage groups consist of only virulent members (Jarvis et al., 1991; Samson and Moineau, 2010), while the P335 quasi-species encompass both virulent and temperate relatives (Braun et al., 1989; Jarvis et al., 1991; Madera et al., 2004; Samson and Moineau, 2010), the latter being able to integrate DNA into the host chromosome to form prophages (Chopin et al., 2001; Kelly et al., 2013).

Strains harboring prophages in the genomes are referred to as lysogenic. Such phenomenon is quite common among cheese starter strains (Davidson et al., 1990). Prophage genes have been shown recently to constitute some 1.75% and 0.65% of the genomes of *Lc. lactis* and *Le. mesenteroides*, respectively (Erkus et al., 2013). Almost all sequenced *Lc. lactis* strains have at least one full-length prophage integrated in the genome (Kelly et al., 2013).

Prophages can be released spontaneously and in the presence of inducers (Huggins and Sandine, 1977; Lunde et al., 2003; Maniloff, 2012; Meister and Ledford, 1979), with the frequency of spontaneous induction being lower than that driven by inducers (Jarvis, 1989; Reyrolle et al., 1982). Based on previous surveys, Jarvis (1989) summarized that the frequency of lysogeny in dairy starter strains could be in the range of 1–80% and that it depends on, among other factors, the choice of the method used to assess the induced prophages.

Lysogenic starters can serve as the sources of phages to the dairy environment (Bouchard and Moineau, 2000; Durmaz and Klaenhammer, 2000; Reyrolle et al., 1982). Virulent phages can originate from temperate prophages and their remnants (Suárez et al., 2008; Ventura et al., 2007) and may eventually disrupt normal fermentation. Several temperate P335 prophages such as Tuc2009 (Costello, 1988), TP901-1 (Brøndsted et al., 2001), BK5-T (Desiere et al., 2001), phiLC3 (Blatny et al., 2004), r1t (van Sinderen et al., 1996) and phismq86 (Labrie and Moineau, 2007), and the so-called small prophages (satellites) such as bIL310 and bIL312 (Chopin et al., 2001) were induced from dairy starter strains. Although lysogeny is widespread among cheese starters, there is still inadequate knowledge with regards to the overall natural phage and prophage communities of DL-starters.

In this study, we assessed lysogeny in three DL-starters through a combination of conventional and metagenomics approaches. The main objective was to characterize the associated prophages and phages. The three starters are cheese starters for the production of Gouda-like cheeses, chosen among many for being sensitive to different phages. Because of this property, the starters have been used in starter rotation schemes.

2. Materials and methods

2.1. Starters, media and conditions

Three DL-starters (A, B and C) were used for this study. Unless stated, starters were stored at −60 °C and thawed to room temperature prior to trial, M17 agar/broth (Difco, USA) supplemented with 1% lactose or 0.5% glucose (for randomly picked isolates and whole starters, respectively), and $CaCl_2$ and $MgCl_2$ (both 10 mM) was employed for all growth experiments, and cultures were incubated overnight at 30 °C (randomly picked isolates) or 28 °C (whole starters).

2.2. Induction of starter isolates

Overnight colonies from agar plates (spread with 1 mL of $10^7\times$, $10^8\times$ and $10^9\times$ starter dilutions) were picked, transferred to 200 µL broth in microplates and incubated overnight, of which 20 µL was transferred to 180 µL broth for three or more subsequent rounds accompanied by overnight incubations. For the induction experiment, 25 µL of the overnight broth or 25 µL of a lysogenic strain with good inducibility (namely *Lc. lactis* CHCC6052) was transferred to corresponding test and control wells containing 210 µL broth. After 2 h of incubation, test and control samples were supplemented with 0.4 µg mL^{-1} mitomycin C (mC) (Sigma-Aldrich, USA) or 20 µL sterile milliQ H_2O, respectively. OD_{600} was followed every hour using a spectrophotometer (BMG LABTECH, Germany) and after 18 h of growth, the microplates were centrifuged for 2 min at 5000 ×*g*. The supernatant (phage lysate) was stored at 5 °C until use.

2.3. Induction of the whole starters

500 mL M17 broth with glucose added was inoculated with 1% of each of the starters. Cultures were incubated overnight to spontaneously induce the starter strains. To induce the strains by mC, cultures were grown to $OD_{600} = 0.15 \pm 0.05$ and divided into two parts. One part was wrapped with aluminum foil and supplemented with 2 µg mL^{-1} mC, and both parts were incubated. As the mC-induced starters were expected to experience lysis due to disruption of cells by the induced phages, a portion of the culture with no mC was transferred. When the mC induction process was completed, the phage lysate (supernatant) was obtained by 15 min centrifugation of spontaneously and mC-induced cultures at 28,000 × *g* in the presence of 1 M NaCl. The phages in the phage lysates were precipitated by 10% polyethylene glycol 6000 (Sigma-Aldrich) and pelleted by 15 min centrifugation at 15,000 × *g*. A solution of 1.3 g mL^{-1} CsCl in SM buffer (18.0 mM $MgSO_4·7H_2O$, 0.1 M NaCl, 0.05 M Tris-HCl pH 8.0) was used to resuspend the pellet, which was purified by 2½ h of ultracentrifugation (106,750 × *g*, 15 °C) in the presence of a lower gradient of 1.5 mL CsCl solution in SM buffer (density = 1.7 g mL^{-1}). The CsCl purification step was repeated and phages collected from the portion of the gradient containing visible bands using a needle installed on a syringe and stored at −20 °C until needed.

2.4. Test of sensitivity with indicator strains

Sensitivity of indicator strains to the induced phage lysates was determined by the double-layer plaque assay (Lillehaug, 1997), using 0.4% agarose (Life Technologies, USA) in the top layer.

2.5. Propagation of phage isolates and DNA preparation

Host-identified phage isolates (2H4, 4E10, 3C05-2, 3B09-1 and 3G11-2) were propagated from a single plaque essentially as previously described (Emond et al., 1997; Jarvis, 1978). Measurement of the phage titers (using spot test) and extraction of DNA were carried out essentially as described by Mazzocco et al. (2009) and Moineau et al. (1994), respectively.

Host-unidentified phage lysates (3D01-1, 3C03-1, 2H07, 4B09, 2H11, A11-1, G12-2, D12-2, H12-2 and A12-2) were prepared as follows: ∼300 µL of the overnight culture of the carrier host was inoculated into 24 mL broth and incubated until $OD_{600} \approx 0.2$. The culture was induced by 2 µg mL^{-1} mC and incubated. At $OD_{600} \leq 0.1$, the samples were stored overnight at 4 °C and treated according to the procedures described by Sambrook and Russell (2001). DNA was extracted essentially as described by Durmaz and Klaenhammer (2000).

2.6. DNA preparation from whole starter phage lysates

Prior to DNA extraction, phage lysates induced from the whole starters were dialyzed against dialysis buffer as described by Sambrook and Russell (2001) and treated with 50 units mL^{-1} DNase I (Sigma-Aldrich) for at least 30 min. DNase I was inactivated by 10 mM EDTA and 1% SDS (both Sigma-Aldrich). After incubation for 1 h at 55 °C with

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