International Journal of Food Microbiology 201 (2015) 47–51

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Short communication

Investigating the requirement for calcium during lactococcal phage infection

Jennifer Mahony [a], Denise M. Tremblay [c], Simon J. Labrie [c], Sylvain Moineau [c], Douwe van Sinderen [a,b,*]

[a] School of Microbiology, University College Cork, Cork, Ireland
[b] Alimentary Pharmabiotic Centre, University College Cork, Cork, Ireland
[c] Département de Biochimie, Microbiologie et Bio-Informatique, Faculté des Sciences et de Génie, GREB & Félix d'Hérelle Reference Center for Bacterial Viruses, Faculté de Médecine Dentaire, Université Laval, Québec City, Québec, Canada, G1V 0A6

ARTICLE INFO

Article history:
Received 16 October 2014
Received in revised form 5 February 2015
Accepted 16 February 2015
Available online 21 February 2015

Keywords:
Bacteriophage
Lactococcus
Calcium
Dairy
Fermentation

ABSTRACT

Calcium is widely used in the study and successful propagation of virulent bacteriophages infecting lactic acid bacteria; however, it has not been assessed if and why this divalent cation is required for the infection process. Lactococcal phages are a persistent problem in the calcium-rich dairy environment and therefore were used as the model for this study. Using representative members of nine of the currently recognized ten lactococcal phage groups, encompassing phages of the *Podoviridae* and *Siphoviridae* families, we present data to suggest that calcium is not an explicit requirement for many of these phages. However, calcium expedited the pace of the lytic cycle for certain phages. Additionally, for calcium-dependent phages belonging to the 936 siphogroup, we could substitute this cation with magnesium or manganese, indicating that these phages are more adaptable than lactococcal phages of other groups. We postulate that the ability of phages to adapt to their environment and to harness the available mineral content may ultimately decide the success of a given phage infection. This may explain, in part, why 936 phages are one of the most frequently isolated phages in the dairy industry.

1. Introduction

Calcium and other divalent cations have long been associated with successful infection of a bacterial cell by a phage. It has been established for certain phages of *Escherichia coli*, *Bacillus subtilis* and *Lactobacillus* spp., among others, that phage adsorption can occur in the absence of calcium (Lu et al., 2003; Steensma and Blok, 1979; Watanabe and Takesue, 1972), while the transfer of DNA across the cytoplasmic membrane is less efficient in its absence (Bonhivers and Letellier, 1995). Additionally, the requirement of calcium for late stages of development and assembly of progeny phages by the *Pseudomonas* phage PM2 has also been demonstrated (Snipes et al., 1974). On the other hand, *Lactobacillus plantarum* phage JL-1 and *Lactobacillus brevis* phage SA-C12 do not require calcium for their infectious cycle, indicating that certain phages do not require calcium to produce progeny phages (Deasy et al., 2011; Lu et al., 2003). It has also been suggested that magnesium, manganese or another divalent cation may substitute the essential role of calcium (Cvirkaite-Krupovic et al., 2010), while in other cases calcium is specifically required for plaque formation and cannot be replaced by such alternatives (Watanabe and Takesue, 1972).

Lactococcal phages are primarily isolated from dairy environs and it is therefore not surprising that calcium is beneficial for their amplification. Lactococcal phages are currently classified into ten groups based on comparative genomics and morphological profiling (Deveau et al., 2006). Of these, three groups account for the majority of lactococcal phage isolates from failed industrial milk fermentations; namely the virulent 936 and c2 phage groups, and the P335 phage group, whose members may be temperate or virulent (Madera et al., 2004; Moineau et al., 1996; Murphy et al., 2013; Raiski and Belyasova, 2009; Szczepanska et al., 2007).

Members of the lactococcal 936 phage group are known to require calcium for efficient production of progeny phages, while it is not essential for adsorption or DNA ejection (Geller et al., 2005; Veesler et al., 2012). Interestingly, the involvement of calcium in the conformational change of the baseplate of the phage p2 (936 group) was demonstrated, where the presence of this cation led to a 200° baseplate downward rotation, in a position conducive to host recognition and its biologically active conformation (Sciara et al., 2010). The baseplate of the rare lactococcal phage 1358 was also shown to have two conformations similar to phage p2 (Spinelli et al., 2014). However the role of calcium, if any, in the conformational change has not been demonstrated as both conformations were detected under identical conditions. The adsorption of phage 1358 to its bacterial host (but not plaque formation) is calcium-independent (Spinelli et al., 2014). Recently, several members of the 936 phage group and P335 group phages Tuc2009 and Q33 have been reported to require calcium for plaque formation and a

* Corresponding author at: School of Microbiology, University College Cork, Cork, Ireland. Tel.: +353 21 4901365; fax: +353 21 4903101.
E-mail address: d.vansinderen@ucc.ie (D. van Sinderen).

http://dx.doi.org/10.1016/j.ijfoodmicro.2015.02.017
0168-1605/

direct correlation between the concentration of calcium and the efficiency of plaquing was observed (Veesler et al., 2012). In contrast, other members of the P335 phage group exhibited no requirement for calcium.

Calcium is routinely added to cultivation media for the propagation and enumeration of lactococcal phages although it may not be necessary (Chopin et al., 2007; Dupuis and Moineau, 2010; Forde et al., 1999; Lillehaug, 1997; Samson and Moineau, 2010). Because lactococcal phages are considerable risk to cheese manufacturers (Samson et al., 2013), "Phage inhibitory medium" (PIM) has been designed. These whey-based bulk starter media contain phosphates to prevent phage proliferation through phosphate-mediated removal of soluble divalent cations (particularly calcium). Its efficacy has been variable, indicating that perhaps not all phages require calcium for infection (Mahony et al., 2014; Richardson et al., 1977).

In this study, we aimed to define the requirement for calcium by representative and prototypical members of each of the currently recognized lactococcal phage groups.

2. Materials and methods

2.1. Phages and bacterial strains

The lactococcal strains and phages used in this study are listed in Table 1. Most of the phages are all available through the Félix d'Hérelle Reference Center for Bacterial Viruses (www.phage.ulaval.ca). Lactococcal host strains were grown without agitation at 30 °C in M17 broth (Oxoid) supplemented with 0.5% glucose.

2.2. Phage propagation and adsorption assays

Phages were propagated on appropriate *Lactococcus lactis* host strains, which had been grown to an approximate optical density (600 nm) of 0.15 in 10 ml M17 broth supplemented with 0.5% glucose. Calcium chloride was added to a final concentration of 10 mM prior to infection of the bacterial culture with approximately 10^8 plaque forming units (pfu) of the relevant phages, which was then followed by incubation at 30 °C or room temperature until lysis had occurred. The lysates were centrifuged and filtered (0.45 μm) to remove any residual bacterial debris and stored at 4 °C. Adsorption assays were performed as previously described (Garvey et al., 1996).

2.3. Calcium-dependent plaque formation & calcium replacement assays

To determine if the phages are dependent on calcium for successful infection of its host, plaque assays were performed in the presence of a range of calcium concentrations (0, 0.1, 0.5, 1, 5 and 10 mM Ca^{2+}) or in the presence of 10 μM and 100 μM EDTA to chelate divalent cations that may be available in the medium. Plaque assays were performed using an adapted version of the previously described double-agar method (Lillehaug, 1997). The relevant level of calcium or EDTA was added to both the M17 solid and semi-solid agar supplemented with 0.5% glucose and 0.5% glycine, and plates were incubated overnight at 30 °C. Plaques were enumerated and recorded as number of plaque forming units per ml of phage lysate (pfu/ml). For phage KSY1, exponentially growing cells were used ($OD_{600} = 0.3$), the media was supplemented with 0.1 μg/ml of penicillin and Petri dishes were incubated at 20 °C for seven days to improve plaque visibility. Penicillin weakens the cell wall, thus increasing plaque size and visibility. We also demonstrated that penicillin does not influence the titer of the lactococcal phage p2 (data not shown). The addition of penicillin was necessary only for KSY1 to provide reproducible plaque formation and was not required for all other phages assessed in this study. Calcium replacement assays were performed using plaque assays in the presence of 10 mM $MgCl_2$ or $MnCl_2$ in place of $CaCl_2$. This was performed to assess if calcium is specifically required by phage 1706, which was identified as calcium-dependent for plaque formation in this study. A similar analysis was performed for Tuc2009, Q33, sk1, p2 and bIL170, which had previously been identified as calcium-dependent (Veesler et al., 2012).

2.4. Lysis-in-broth assay

To ascertain if calcium ions influence the timing of the phage cycle, lysis-in-broth assays were performed by infecting the relevant culture with each of the phages in the presence or absence of 10 mM calcium chloride at a multiplicity of infection of 1.0. Lysis was monitored using OD_{600} readings at 30-minute intervals for 180 min post-infection. All assays were performed in triplicate.

3. Results & discussion

3.1. Defining the requirement for calcium in lactococcal phage infection

Plaque assays involving a range of calcium chloride concentrations from 0 to 10 mM, or the presence of the chelating agent EDTA were performed. Out of the 8 phages tested, only 1706 required calcium for plaque formation (Table 2). Indeed, phage 1706 was unable to produce plaques with medium containing less than 10 mM $CaCl_2$. The two c2-like phages (c2 and 952), as well as 949, Q54 and P087, infected their host with comparable efficiency in the presence or absence of calcium or in media containing EDTA although plaque size and visibility were affected in low calcium and in the presence of EDTA (data not shown). Plaque formation of phage KSY1 was also shown to be calcium-independent, although the phage seems to be slightly inhibited in the presence of 100 μM of EDTA with a slight reduction in the efficiency of plaquing (Table 2).

While plaque formation may be largely unaffected in the absence of calcium for most of the phages tested, the possibility of a role for calcium in phage development in broth was assessed. To this end, lysis-in-broth studies were performed with the same 8 lactococcal phages

Table 1
Lactococcal strains and phages used in this study.

Phage	Species	Reference	Lactococcal host strain	Reference
sk1	936	Higgins et al. (1988)	NZ9000	Kuipers et al. (1998)
p2	936	Higgins et al. (1988)	NZ9000	Kuipers et al. (1998)
bIL170	936	Crutz-Le Coq et al. (2002)	IL1403	Chopin et al. (1984)
Tuc2009	P335	Arendt et al., (1994)	UC509.9	Costello (1988)
Q33	P335	Moineau et al. (1996)	SMQ86 (UL8)	Moineau et al. (1992)
949	949	Jarvis (1977)	ML8	Jarvis and Jarvis (1981)
KSY1	KSY1	Saxelin et al. (1979)	IE-16	Deveau et al. (2006)
P369	P034	Braun et al. (1989)	F7/2	Lembke et al. (1980)
c2	c2	Sanders and Klaenhammer (1980)	NZ9000	Kuipers et al. (1998)
952	c2	Garvey et al. (1996)	NZ9000	Kuipers et al. (1998)
Q54	Q54	Deveau et al. (2006)	SMQ-562	Deveau et al. (2006)
P087	P087	Lembke et al. (1980)	C10	Lembke et al. (1980)
1706	1706	Deveau et al. (2006)	SMQ-450	Deveau et al. (2006)