Large-scale dairy fermentations can be seriously compromised by bacteriophages infecting lactic acid bacteria (LAB), and particularly *Lactococcus lactis*, which is a commonly used starter culture for cheese making (Garneau & Moineau, 2011). Lactococcal phages are currently classified in 10 groups, eight of them belonging to the *Siphoviridae* family of the order *Caudovirales*. Those of the c2, 936 and P335 groups are the most commonly found in dairy plants (Deveau et al., 2006).

A considerable effort has been made to improve and select phage-resistant starter strains based on the knowledge of resistance mechanisms developed by LAB and a better understanding of phage–host interactions (Sturino & Klaenhammer, 2006). Described mechanisms of phage resistance expand from blocking adsorption, restriction/ modification enzymes, to abortive infection systems that interfere with phage DNA replication, morphogenesis and release. Clustered regularly interspaced short palindromic repeats (CRISPRs) located in the genomes of host bacteria together with a group of associated proteins can also confer resistance to phages (Labrie et al., 2010). Recently, the response to phage infection in *L. lactis* has been approached by genome-wide transcriptomics (Fallico et al., 2011; Ainsworth et al., 2013). *L. lactis* appears to sense bacteriophage infection as a perturbation of its cell envelope and mounts a response targeted to the cell wall, activating regulators of the cell envelope stress response such as the two-component system CesSR.

One of the members of the CesSR regulon in *L. lactis* is the *ftsH* gene (Martínez et al., 2007). *ftsH* encodes a conserved AAA (ATPase associated with various cellular activities)-type membrane protease involved in stress response and protein quality control (Ito & Akiyama, 2005; Narberhaus et al., 2009). In this work, we analysed the impact of the *ftsH* null mutation on the life cycle of the P335 temperate phage TP712, which is able to infect and lysogenize *L. lactis* MG1363 (Gasson, 1983) and derivatives thereof. The complete TP712 nt sequence has been determined (GenBank accession number AY766464) essentially as described by Wegmann et al. (2012).

Preliminary experiments revealed that in standard plaque assays (Lillehaug, 1997), the efficiency of plaquing (EOP) of this phage on an available non-polar and in-frame *L. lactis* mutant lacking *ftsH* (Pinto et al., 2011) was reduced to $1.5 \times 10^{-5}$ when compared to its parent *L. lactis* NZ9000. Upon complementation with the plasmid pUK200 :: *ftsH*, in which *ftsH* from *L. lactis* MG1363 is under the control of the inducible nisin promoter (P*nisA*), plaque formation was restored (Fig. 1a, b), demonstrating complementation of the *ftsH* mutation. To discard any other unintended mutations, a new *L. lactis* NZ9000 Δ*ftsH* strain was created by introducing an in-frame deletion of the internal 1.48 kbp HindIII fragment of *ftsH*, using the thermosensitive plasmid pGhost9 (Maguin et al., 1996). Resistance to TP712 was also consistently observed. Infection by other lactococcal phages such as c2 and sk1, belonging to the c2 and 936 groups, respectively, was not compromised with EOPs of 1.1 and 0.9.
respectively (data not shown). Four phages from the P335 group were also tested, but they did not infect the WT host *L. lactis* NZ9000.

Based on the role of FtsH in shaping the membrane composition in Gram-negative micro-organisms (Katz & Ron, 2008) and surface properties in *L. plantarum* (Bove et al., 2012), we postulated that FtsH could be involved in adsorption, acting either as a phage receptor itself or altering the availability of receptors. Adsorption assays were carried out by incubating bacterial strains and phage TP712 at an m.o.i. of 0.001 for 10 min at 30 °C, and then counting the non-adsorbed phages that were collected after centrifugation (Madera et al., 2003). No substantial differences were observed between the WT *L. lactis* NZ9000 and the ΔftsH mutant, with percentages of adsorbed phages from 40 % to 80 % in both strains.

To elucidate if FtsH was required for the delivery of DNA into the bacterial cell, the tagged bacteriophage STTP712 was constructed by deleting the region stretching from position 15717 to 17885 [including the IS712 element (15724–17885) and orf31-orf32 of TP712 encoded thereon] and inserting the spectinomycin-resistance gene (*spec*) from *Enterococcus faecalis* into the adjacent SpeI restriction site. Replacement was thought to be the safest way to avoid getting defective STTP712 phage mutants since TP712 may follow a headful packaging DNA model based on its sequence relatedness to the group of Sfi11-like pac site *Siphoviridae*. The replacement did not compromise phage viability. After STTP712 induction with mitomycin C (mitC), the cells lysed at the same rate as those carrying TP712 and growth of *L. lactis* STTP712 lysogens was not affected either (data not shown). STTP712 lytic plaques were produced in the wild-type strain and were absent in the ΔftsH mutant. On the other hand, the same percentage of lysogens (0.02 %) in both *L. lactis* strains was scored, as judged by the development of spectinomycin-resistant colonies after infection (Fig. 1c, d).

FtsH contributes to the activation of the lytic cycle of phage λ in *E. coli* by regulating the levels of the transcription factor CII and Xis (Shotland et al., 1997; Jeffers & Gottesman, 1998). Thus, a probable scenario could be that TP712 gets locked in the lysogenic state in *L. lactis* ΔftsH because the ftsH mutation inhibits prophage activation or replication of the TP712 DNA. To test this hypothesis, the ftsH mutation was introduced in a TP712 lysogenic strain and *L. lactis* UKLc10 ΔftsH TP712 was generated. After addition of mitC to exponentially growing cultures, a sharp decrease in the optical density at 600 nm (OD<sub>600</sub>) was observed in the WT cultures, indicative of prophage induction and release (Fig. 2), while only a slight decrease was initially noted in the ΔftsH mutant, but lysis did not progress any further. When complemented with the plasmid pUK200::ftsH, lysis was partially restored (Fig. 2a). The phage titre in the culture supernatants after 3 h of mitC induction was at least three orders of magnitude

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**Fig. 1.** Plaque assays of the bacteriophage TP712 on *L. lactis* strains. (a) Absence of plaque formation on *L. lactis* NZ9000 ΔftsH and (b) complementation in *L. lactis* NZ9000 ΔftsH pUK200::ftsH induced with nisin (0.1 ng ml<sup>−1</sup>). (c) Absence of plaques of the (*spec*)-tagged bacteriophage STTP712 on *L. lactis* NZ9000 ΔftsH. (d) *L. lactis* NZ9000 ΔftsH lysogens grown on GM17 supplemented with spectinomycin (100 μg ml<sup>−1</sup>) after infection with STTP712. (e, f) Presence of viable and infective TP712 virions in the cytoplasm of non-induced (e) and of mitC-induced (1 μg ml<sup>−1</sup>) *L. lactis* UKLc10 ΔftsH TP712 (f). In (e) and (f), *L. lactis* cells were lysed by the addition of lysozyme.
higher in *L. lactis* UKLc10 TP712 compared to its ∆ftsH counterpart with titres over 10^6 and 10^3 p.f.u. ml⁻¹, respectively. Additionally, a sharp increase of phage DNA following the protocol previously described (Sobero´ n et al., 2007), using primers TP03 5’-CGCTGACAGTTGACTGATG-3’ and TP04 5’-GCCAACGACTCGTTTAGAC-3’ annealing within the major head protein gene (orf42). Therefore, a putative role of FtsH in activating the lytic cycle or in phage DNA replication was ruled out.

MitC-induced *L. lactis* UKLc10 ∆ftsH TP712 cells were observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The non-induced ∆ftsH strain had the usual morphology and smooth surface of a Gram-positive ovococcus without any detectable alteration (Fig. 3a, c). On the contrary, mitC-induced cells become elongated with several growth rings in the same cell (Fig. 3b, d, e). Lysis occurred only in a few cells, somewhat in a localized fashion, at the septum area where the peptidoglycan layer is thinner or partially digested. Otherwise, cell walls remained intact. The lysis pattern was remarkably different in WT cells that showed a few cells, somewhat in a localized fashion, at the septum area where the peptidoglycan layer is thinner or partially digested. Otherwise, cell walls remained intact. The lysis pattern was remarkably different in WT cells that showed a typical ‘slicing’ of the host cells into rings and end caps (Fig. 3f). On the other hand, assembled viral particles inside the cells were observed (Fig. 3). These results show that in absence of FtsH, virions appeared to assemble properly and pile up inside the cell.

To verify if these assembled particles were infective, mitC-induced and non-induced cells were treated with lysozyme (1 mg ml⁻¹) in 0.01 M Tris/HCl pH 8 buffer at 37 °C for 30 min to hydrolyse the peptidoglycan. When the cytosolic content was plated, over 3 × 10^2 p.f.u. ml⁻¹ were counted in samples from non-induced cells (i.e. spontaneous induction), while confluent lysis was observed in the case of mitC-induced cells (Fig. 1e, f). Hence, absence of FtsH inhibits release of the phage progeny to such an extent that cell lysis in infected broth cultures, as well as lytic plaques on *L. lactis* ∆ftsH lawns, are no longer perceptible.

Dependence on the host FtsH to trigger lysis after phage infection has not been described so far. Nevertheless, the T4 antiholin R1 is degraded by the host protease DegP (Tran et al., 2013). FtsH might be tuning host lysis by regulating the levels of the TP712 (anti)holin and/or endolysin. Interestingly, TP712 holin seems to be structurally similar to the SAR-containing holin of phage 21 (Park et al., 2006) and recently a SAR domain has been identified in homologues of the TP712 endolysin (Oliveira et al., 2013). Any of these SAR domains could be tentatively targeted and processed by FtsH. However, because very little is known about the role of FtsH in *L. lactis*, it is also possible that absence of ftsH, although tolerated by *L. lactis*, may have physiological consequences (e.g. altered peptidoglycan structure) that impair phage lysis. FtsH has been involved in the transcriptional regulation of *pbpE*, encoding the D,D-endopeptidase PBP4* in *Bacillus subtilis* (Zellmeier et al., 2003). Upregulation of *pbpE* makes *Bacillus* peptidoglycan less susceptible to mutanolysin (Palomino et al., 2009). These and other likely hypothesis should be addressed in the future to unravel the molecular mechanisms behind phage resistance in the ∆ftsH background. This knowledge could tentatively contribute to generate or select for new phage resistant *L. lactis* strains without compromising their performance as starters.

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**Fig. 2.** Induction of the lysogenic *L. lactis* UKLc10 TP712 strains. (a) Growth after prophage induction with mitC (1 µg ml⁻¹) at time 0. *L. lactis* UKLc10 TP712 (■), UKLc10 ∆ftsH TP712 (×) and UKLc10 TP712 pUK200 ∆ftsH induced with nisin (0.1 ng ml⁻¹) (○). Means and standard deviations of three independent cultures are represented. (b) Replication of bacteriophage TP712 DNA determined by qPCR. *L. lactis* UKLc10 TP712 (■) and *L. lactis* UKLc10 ∆ftsH TP712 (○), induced with mitC (2 µg ml⁻¹) at time 0 (filled symbols) and not induced (open symbols). C_t values are relative to each C_t at time 0. Negative values (−∆C_t) are displayed to visualize better the increase of DNA, i.e. lower C_t values after induction. Means and standard deviations of two independent assays are represented.
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Fig. 3. Micrographs of lysogenic L. lactis strains after 4 h of prophage induction. Scanning (a, b) and transmission (c–e) micrographs of non-induced (a, c) and mitC-induced (1 μg ml⁻¹) (b, d, e) L. lactis UKLc10 ΔftsH TP712. (f) Scanning micrograph of mitC-induced (2 μg ml⁻¹) WT L. lactis FI7274, a TP712 lysogen derivative of L. lactis MG1363 (ftsH +). Bars, 1 μm (a, b), 500 nm (c), 100 nm (d, e) and 200 nm (f).
References


