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The *ltp* gene of temperate *Streptococcus thermophilus* phage TP-J34 confers superinfection exclusion to *Streptococcus thermophilus* and *Lactococcus lactis*

Xingmin Sun [1], André Göhler, Knut J. Heller *, Horst Neve

Institute for Microbiology, Federal Research Center for Nutrition and Food, PO Box 6069, D-24121 Kiel, Germany

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Abstract

The *ltp* gene, located within the lysogeny module of temperate *Streptococcus thermophilus* phage TP-J34, has been shown to be expressed in lysogenic strain *S. thermophilus* J34. It codes for a lipoprotein, as demonstrated by inhibition of cleavage of the signal sequence by globomycin. Exposure of Ltp on the surface of *Lactococcus lactis* protoplasts bearing a plasmid-encoded copy of *ltp* has been demonstrated by immunogold labeling and electron microscopy. Expression of *ltp* in prophage- and plasmid-cured *S. thermophilus* J34-6f interfered with TP-J34 infection. While plating efficiency was reduced by a factor of about 40 and lysis of strain J34-6f in liquid medium was delayed considerably, phage adsorption was not affected at all. Intracellular accumulation of phage DNA was shown to be inhibited by Ltp. This indicates interference of Ltp with infection at the stage of triggering DNA release and injection into the cell, indicating a role of Ltp in superinfection exclusion. Expression of *ltp* in *L. lactis* Bu2-60 showed that the same superinfection exclusion mechanism was strongly effective against phage P008, a member of the lactococcal 936 phage species: no plaque-formation was detectable with even 10^9 phage per ml applied, and lysis in liquid medium did not occur. In *Lactococcus* also, Ltp apparently inhibited phage DNA release and/or injection. Ltp appears to be a member of a family of small, secreted proteins with a 42 amino acids repeat structure encoded by genes of Gram-positive bacteria. Some of these homologous genes are part of the genomes of prophages.

Keywords: Temperate *S. thermophilus* phage TP-J34; *ltp* gene; Lipoprotein; Superinfection exclusion; *Lactococcus lactis* phage P008

Introduction

In contrast to lactococci and lactobacilli, lysogeny is a rare event in *Streptococcus thermophilus* (Bruttin et al., 1997; Josephsen and Neve, 2004). *S. thermophilus* J34 from our strain collection was originally isolated from yogurt and has been shown to be lysogenic for a temperate phage designated TP-J34 (Neve et al., 1998, 2003). Upon induction with either UV-light or mitomycin C, phage particles were released, which were mainly defective showing DNA-filled heads with no tails attached (Neve et al., 2003). It appears that a particular structure of a gene encoding a tail protein is responsible for this defect (B. Rabe, K.J. Heller and H. Neve, unpublished results), since a plaque-forming, morphologically intact mutant, TP-J34L, was isolated, which did not show this particular structure (Neve et al., 2003). This mutant was very helpful for further studies of TP-J34 physiology, because it allowed quantitative determination of plaque forming units.

Phage TP-J34 confers upon its host strain J34 a lysogenic conversion phenotype. While lysogenic strains grow homogeneously in broth, prophage-cured derivatives reveal a clumping phenotype and sediment at the bottom of tubes when left without shaking for several hours (Neve et al., 2003). In addition, *S. thermophilus* J34 is astonishingly refractory to phage infection.

* Corresponding author. Fax: +49 431 609 2306.
E-mail addresses: Xingmin_Sun@brown.edu (X. Sun), knut.heller@bfel.de (K.J. Heller).
[1] Present address: Department of Molecular Biology, Cell Biology, Biochemistry Box G-J36, Brown University, Providence, RI 02912, USA. Fax: +1 401 863 1348.

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The *ltp* gene (lipoprotein of temperate phage TP-J34, i.e., *orf142*) (Neve et al., 1998) is located on TP-J34 DNA in an operon together with three other genes. The operon starts with a gene encoding the putative repressor needed for maintenance of the lysogenic state, followed by a gene encoding a protein exhibiting a metallo-proteinase motif. The third gene is *ltp*, and the last one is the *int* gene encoding the putative phage integrase. The *ltp* gene codes for a putative protein (Ltp) of 142 amino acids (aa) with a prolipoprotein signal peptide (von Heijne, 1989) and a calculated molecular weight of 15.8 kDa. Recently, Bruttin et al. (1997) and McGrath et al. (2002) showed that genes conferring superinfection exclusion are located on the DNA of an *S. thermophilus* and a lactococcal temperate phage, respectively, at exactly the same topological position as *ltp* in *S. thermophilus.*

Phage-encoded lipoproteins are rather rare. In coliphage lambda, two lipoproteins synthesized during lysogeny have been described: the one encoded by *bor* is involved in serum resistance of lysogenic cells (Barondess and Beckwirth, 1995), and the one encoded by *rz1* is involved in lysis (Kedzierska et al., 1996). Two mycobacterial prophages have been described to induce synthesis of lipoproteins, which may be involved in virulence (Armoa et al., 1995) or in enhanced immunogenicity (Voelker and Dybvig, 2004), respectively. The Cor proteins of the lambdoid, FhuA-dependent phages phi80, N15, HK022, mEp167 (Uc-Mass et al., 2004), and T1 (Roberts et al., 2004) confer a superinfection exclusion (Uc-Mass et al., 2004) or lysogenic conversion phenotype (Vostrov et al., 1996) to cells carrying the prophages. Although not explicitly shown, they appear to be lipoproteins, as their amino acid sequences exhibit typical signal sequences and a lipoprotein box (von Heijne, 1989) with the lipid-modified cystein residue at the signal sequence cleavage site. The only lipoprotein shown to be synthesized during infection by a lytic phage is the phage T5 Llp protein, involved in lytic conversion-mediated phage resistance of the infected cell (Decker et al., 1994; Braun et al., 1994; Pedruzzi et al., 1998). Recently, two genes encoding lipoproteins were detected within the lysogeny module of two *Lactobacillus* prophages (Ventura et al., 2004a, 2004b).

In this communication, we show by globomycin inhibition of its processing that Ltp is indeed a lipoprotein, which is synthesized in lysogenic *S. thermophilus* J34. Since expression of *ltp* appeared to interfere weakly with phage infection in *S. thermophilus*, we propose that Ltp is involved in superinfection exclusion. Furthermore, Ltp is shown to confer a strong inhibitory effect against phage P008 infecting a different lactic acid bacterial species, namely *L. lactis*.

Results

Cloning and expression of ltp

The *ltp* gene was cloned into the pET-11d vector under the control of the *lac* promoter to yield pXMS1. pXMS1 was transformed into *E. coli* strain BL21(DE3). A high level of expression was achieved after 3 h of induction with 1 mM IPTG in this strain. Expression of *ltp* yielded a protein of ca. 17 kDa, as monitored by SDS-PAGE (not shown).

The deduced aa sequence of the *ltp* gene product suggests that it is a lipoprotein (Neve et al., 1998). The calculated molecular mass of the mature, lipid modified Ltp should be ca. 14 kDa. The discrepancy to the 17 kDa detected may result from lipid modification and/or some as yet unidentified secondary structures of the Ltp polypeptide. Besides the signal sequence cleaved during secretion, no other part of the amino acid sequence of Ltp shows characteristics compatible with membrane-spanning.

In *E. coli*, isopycnic sucrose gradient centrifugation of total cell walls showed that Ltp was predominantly associated with the outer membrane fraction, only limited amounts were recovered from the inner membrane fraction (data not shown). The outer membrane fraction of *E. coli* BL21(DE3)(pXMS1) was subjected to SDS-PAGE, the protein band corresponding to Ltp was excised from the gels and used for immunization and subsequent raising of a polyclonal antiserum.

Ltp is a lipoprotein expressed during lysogeny in S. thermophilus

To demonstrate that Ltp was a lipoprotein, globomycin – an inhibitor of the signal peptidase specific for lipoprotein processing (Regue and Wu, 1988) – was added to growing cultures of *E. coli* BL21(DE3)(pXMS1). Total cellular proteins were analyzed by Western blot, and a protein band with a molecular mass size ca. 2–3 kDa larger than the 17-kDa Ltp band was detected by Ltp-specific antibodies in *E. coli* (Fig. 1). For *E. coli*, it can be seen that the extent of inhibition of processing was dependent on the concentration of globomycin.

The antibodies raised against purified Ltp were tested on lysogenic strain J34 and its prophage-cured derivative J34-6 by Western blot analysis. Lysogenic strain J34 expressed a ca. 17-kDa protein band, corresponding to the one of the *E. coli* control cells (Fig. 1). No protein band was detected with the Ltp-specific antibodies in TP-J34-cured strain J34-6 (data not shown). Upon addition of globomycin, inhibition of processing of Ltp was also shown for *S. thermophilus* J34 (Fig. 1). These results demonstrate that *ltp* is expressed during lysogeny. However, *S. thermophilus* J34 appeared to be more sensitive to

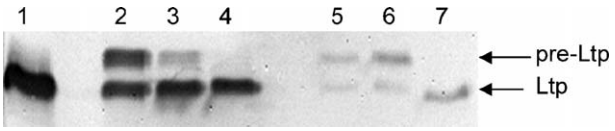

Fig. 1. Inhibition of Ltp-processing by globomycin. *E. coli* BL21(DE3) (pXMS1) cells were grown in LB-medium supplemented with 100 μg Ap per ml at 37 °C (lanes 1–4), *S. thermophilus* J34 cells were grown in *th*LM17 medium supplemented with 1 μg Em per ml at 40 °C (lanes 5–7). Globomycin, dissolved at 5 mg/ml in methanol, was added at final concentrations of 25 μg/ml (lanes 3 and 6) and 50 μg/ml (lanes 2 and 5). For control, 10 μl methanol was added (lanes 4 and 7). Ltp was detected in whole cell extracts by Western blotting. Lane 1 shows gel purified Ltp isolated from outer membranes of *E. coli* BL21(DE3) (pXMS1) and used for antibody production.