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Identification of the Lower Baseplate Protein as the Antireceptor of the Temperate Lactococcal Bacteriophages TP901-1 and Tuc2009

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The first step in the infection process of tailed phages is recognition and binding to the host receptor. This interaction is mediated by the phage antireceptor located in the distal tail structure. The temperate *Lactococcus lactis* phage TP901-1 belongs to the P335 species of the *Siphoviridae* family, which also includes the related phage Tuc2009. The distal tail structure of TP901-1 is well characterized and contains a double-disk baseplate and a central tail fiber. The structural tail proteins of TP901-1 and Tuc2009 are highly similar, but the phages have different host ranges and must therefore encode different antireceptors. In order to identify the antireceptors of TP901-1 and Tuc2009, a chimeric phage was generated in which the gene encoding the TP901-1 lower baseplate protein (*$bppL_{TP901-1}$*) was exchanged with the analogous gene (*$orf53_{2009}$*) of phage Tuc2009. The chimeric phage (TP901-1C) infected the Tuc2009 host strain efficiently and thus displayed an altered host range compared to TP901-1. Genomic analysis and sequencing verified that TP901-1C is a TP901-1 derivative containing the *$orf53_{2009}$* gene in exchange for *$bppL_{TP901-1}$*; however, a new sequence in the late promoter region was also discovered. Protein analysis confirmed that TP901-1C contains $ORF53_{2009}$ and not the lower baseplate protein $BppL_{TP901-1}$, and it was concluded that $BppL_{TP901-1}$ and $ORF53_{2009}$ constitute antireceptor proteins of TP901-1 and Tuc2009, respectively. Electron micrographs revealed altered baseplate morphology of TP901-1C compared to that of the parental phage.

The first physical contact between a phage and its bacterial host is a crucial step in the life cycle of the phage. For tailed phages, this initial host interaction is mediated by a so-called antireceptor located in the distal part of the tail. This antireceptor recognizes and interacts with a specific structure or receptor on the surface of the host cell as a prerequisite to the injection of the phage genome. In several *Escherichia coli* phages, e.g., λ, P2, T4, and the T-even-type phages, the phage antireceptors have been identified in tail fibers (20, 26, 48). In contrast to this, the antireceptors of the *E. coli* phages T5 and BF23 have been shown to be located in the tail shaft just above a straight tail fiber (22, 24). Compared to these extensively studied *E. coli* phages, the knowledge of the antireceptors of phages infecting lactic acid bacteria (LAB), and gram-positive bacteria in general, is limited. Most speculations regarding genes encoding host-interacting proteins of LAB phages originate from sequence comparisons among the rapidly increasing number of completely sequenced LAB phages (10, 11, 31, 33, 39). Phage antireceptor proteins of a few *Streptococcus thermophilus* and *Lactococcus lactis* phages have, however, recently been identified. Duplessis and Moineau (12) identified ORF18 as the determinant of host specificity for the *S. thermophilus* phages DT1 and MD4 by the generation of chimeric phages with an altered host range. These chimeric phages were readily propagated on the new host strain, and a C-terminal variable region of *orf18* was found to be responsible for host recognition. In similar studies, Stuer-Lauridsen et al. (44) and Dupont et al. (14) identified receptor binding proteins of the virulent *L. lactis* phages bIL67 and CHL92 of the c2 species and sk1 and bIL170 of the 936 species, respectively. The chimeric phages in those studies displayed an altered host range in plaque assays, but the phages were either partially or completely deficient in their ability to propagate lytically on the new host strain, thus indicating that additional factors may contribute to the host range determination of these phages. Although the receptor binding protein from sk1 was shown to be located at the tip of the phage tail (14), these antireceptors of LAB phages have not yet been assigned to more specific tail structures.

The temperate *L. lactis pac* type phage TP901-1 belonging to the P335 species of the *Siphoviridae* family is among the best-studied LAB phages. The 37.7-kbp genome has been fully sequenced (GenBank accession no. NC_002747) (7), and many aspects of the TP901-1 life cycle have been investigated experimentally (8, 28, 34, 37). We have recently completed a detailed structural characterization of the distal tail of TP901-1, which consists of a double-disk baseplate and a central tail fiber (47). The baseplate was found to assemble onto a conical structure situated below the tail tube, and the proteins BppU

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TABLE 1. Bacterial strains, plasmids, and phages

Bacterial strain, plasmid, or phage	Relevant feature(s)[a]	Reference or source
L. lactis subsp. *cremoris* strains		
901-1	Lysogenic for phage TP901-1	5
3107	Host strain for phage TP901-1	9
UC509.9	Host strain for phage Tuc2009	2
MG1363	Transformational strain	17
CSV52-6	901-1, lysogenic for phage TP901-1*erm*	This study
CSV72-1	3107 + pCSV71-8	This study
CSV75	UC509.9, lysogenic for phage TP901-1C	This study
Plasmids		
pCI372	5.7 kbp; cloning vector; Cm^r	21
pGhost8	5.2 kbp; TS replicon; Tc^r	35
pBC197	13.2 kbp; pCI372::TP901-1 (nt 33,954–2,704) *orf55erm* (nt 35,637); Erm^r Cm^r	30
pCSV27-31	8.4 kbp; pGhost8::TP901-1 (nt 33,954–36,842) *orf55erm* (nt 35,637); Erm^r Tc^r	This study
pCSV71-8	7.9 kbp; pCI372::TP901-1 (nt 29,561–30,390) Tuc2009 (nt 33,461–34,000) TP901-1 (nt 30,897–31,748); Cm^r	This study
Phages		
TP901-1	Temperate phage; isolated following induction of *L. lactis* 901-1	5
Tuc2009	Temperate phage; isolated following induction of *L. lactis* UC509	2
TP901-1*erm*	TP901-1*erm* in $orf55_{TP901-1}$	This study
TP901-1C	Chimeric TP901-1*erm* with $orf53_{2009}$	This study

[a] Abbreviations: Cm^r, chloramphenicol resistance; *erm*, adenine methylase gene from pAMβ1 (6); Erm^r, erythromycin resistance; Tc^r, tetracycline resistance; TS, temperature sensitive. nt, nucleotide position in genome.

and BppL were shown to form the upper and lower baseplate disks, respectively. The baseplate was furthermore demonstrated to be necessary for host infection, and the TP901-1 antireceptor was therefore expected to be located in the baseplate (47). The protein $Tal_{TP901-1}$ was found to share many characteristics with the tail-associated lysin protein Tal_{2009} of phage Tuc2009 (29), and it was shown that $Tal_{TP901-1}$ constitutes the TP901-1 tail fiber, which protrudes below the baseplate (47). Moreover, $Tal_{TP901-1}$, the distal tail protein (Dit), and the tape measure protein were proposed to form a tail assembly initiator complex constituting the conical structure and the tail fiber (38, 47). The individual tail proteins of TP901-1 are thus related to specific tail structures, and TP901-1 is therefore suitable as a model phage for the study of antireceptors of LAB phages. The genomes of TP901-1 and the related *L. lactis* phage Tuc2009 are organized in a similar manner, with particular congruity to gene order and amino acid homology between the encoded tail proteins (7, 31, 42). The phages, however, have different host ranges and are therefore expected to have dissimilar antireceptor proteins.

In this study, we identify antireceptor proteins of TP901-1 and Tuc2009 by the generation of a chimeric TP901-1 phage with an altered host range. This new phage has the TP901-1 lower baseplate gene exchanged with the analogous Tuc2009 gene, and it is shown to efficiently infect the Tuc2009 host strain *L. lactis* UC509.9. Genomic and structural analysis of the chimeric phage confirmed the genetic exchange as well as an alteration of the protein content of the distal tail.

MATERIALS AND METHODS

Bacterial strains, culture conditions, plasmids, and phage preparations. The bacterial strains, plasmids, and phages used in this study are listed in Table 1. *L. lactis* strains were grown in M17 medium (Oxoid Ltd., Basington, Hampshire, England) supplemented with 0.5% (wt/vol) glucose (GM17) (45), or GSB medium (10 g glucose, 10 g beef extract, 5 g yeast extract, 5 g tryptone, 5 g tryptose, 7.2 g sodium-β-glycerophosphate, and 2 g glycine per liter), which is a glucose/glycine modification of LSB (2); 5 μg/ml chloramphenicol, 2 μg/ml tetracycline, or 1 μg/ml erythromycin was added where appropriate. For phage propagations, calcium chloride was added to a final concentration of 5 mM. Preparation of competent cells and electroporation were performed as previously described (47). Strains containing plasmid pGhost8 or derivatives thereof were grown at 28°C, while all other strains were grown at 30°C, unless otherwise stated. The phages TP901-1 wild type (wt) and TP901-1*erm* were induced from their respective lysogenic *L. lactis* 901-1 strains by 3 μg/ml mitomycin C (Sigma-Aldrich, St. Louis, Mo.), while phage Tuc2009 and the chimeric phage TP901-1C were propagated lytically on *L. lactis* UC509.9. Purification of phages from cell lysates by isopycnic centrifugation through cesium chloride density gradients was performed as previously described (47).

DNA technology and sequencing. Phage DNA was isolated from purified phage preparations by phenol-chloroform extraction as described previously for phage λ by Sambrook and Russell (41), while plasmid DNA was isolated with the QIAprep Spin Miniprep or Plasmid Midi kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions, following a 20-min incubation at 37°C with 20 mg/ml lysozyme (Sigma-Aldrich). Phage restriction endonuclease profiles were performed on 1.5 to 2 μg phage DNA, and the digests were carried out according to the enzyme manufacturer's recommendations (New England Biolabs, Beverly, Mass.). Restriction fragments were separated by electrophoresis on 0.7% (wt/vol) agarose (Invitrogen, Carlsbad, Calif.) gels in Tris-amino-EDTA buffer (0.04 M Tris-acetate, 0.02 M EDTA, pH 8.0). PCR amplifications of phage DNA used for cloning were performed with the *Pwo* DNA polymerase (Roche, Mannheim, Germany), while a *Taq* DNA polymerase (Invitrogen) was used for all other amplifications. Water suspensions of *L. lactis* colonies were either boiled or treated with 10 units of mutanolysin (Sigma-Aldrich) prior to colony PCR amplifications. Phage DNA sequences were determined by MWG (Ebersberg, Germany) or with the CEQ 2000 Dye Terminator cycle sequencing kit on a Beckman Coulter CEQ 2000 DNA analysis system (Beckman Coulter Inc., Fullerton, Calif.).

Construction of a TP901-1 prophage mutant encoding an erythromycin resistance marker. A TP901-1 derivative carrying an adenine methylase gene transcribed from a constitutive promoter was constructed as a prophage mutant of a lysogenic *L. lactis* 901-1 strain. The adenine methylase gene, originating from *Enterococcus faecalis* plasmid pAMβ1 (GenBank accession no Y00116) (6), causes bacterial resistance to erythromycin (Erm^r) and is hereafter referred to as *erm*. A TP901-1 EcoRI-EcoRV (genomic position 33,954 to 36,842) DNA fragment containing the 1.2-kbp *erm* gene inserted into $orf55_{TP901-1}$ (genomic posi-

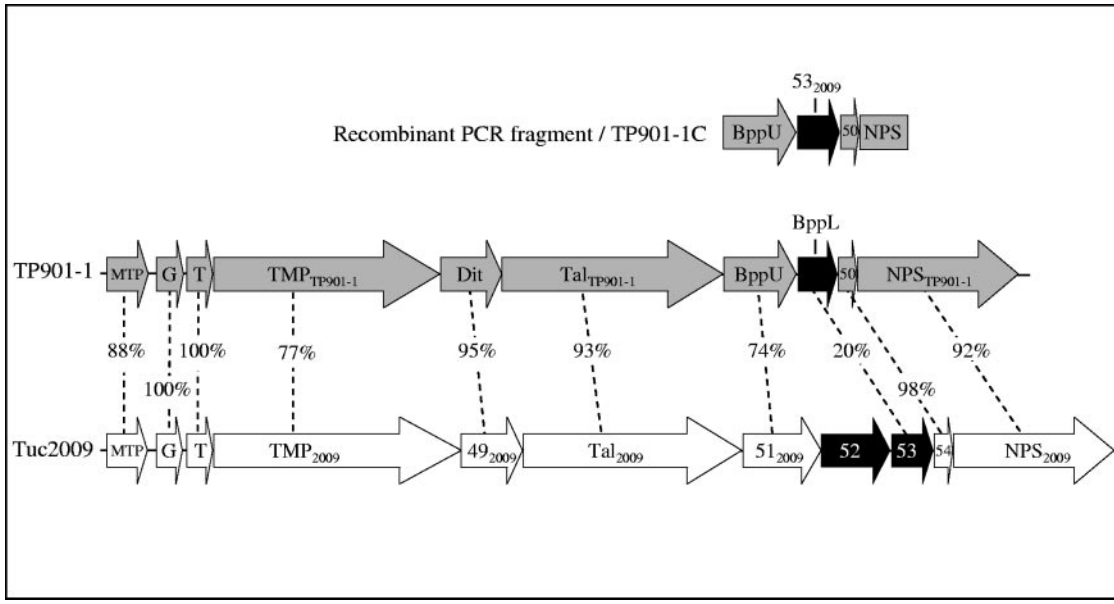

FIG. 1. Tail proteins of the phages TP901-1 and Tuc2009. Percentages of amino acid identity of homologous proteins are indicated between proteins. The values were obtained from a BLASTp search (1) and are stated with respect to the longest protein sequence. Unique proteins, with no or minor sequence similarity to proteins from the opposite phage, are shown in black. Abbreviations: BppL, lower baseplate protein of TP901-1; BppU, upper baseplate protein of TP901-1; Dit, distal tail protein of TP901-1; G, putative equivalent to gpG of λ; MTP, major tail protein; NPS; neck passage structure protein; T, putative equivalent to "gpT" of λ; Tal_{2009}, tail-associated lysin from Tuc2009; $Tal_{TP901-1}$, tail fiber protein of TP901-1. Unnamed proteins are referred to by the numbers of their encoding open reading frames. TP901-1 and 2009 subscripts are omitted where space is limited.

tion 35,637) was subcloned from plasmid pBC197 (30) into the pGhost8 vector, thus forming the construct pCSV27-31. Following transformation of *L. lactis* MG1363 and subsequent plasmid purification, pCSV27-31 was introduced into the TP901-1 lysogenic strain *L. lactis* 901-1 by transformation. The *erm* gene was finally transferred from pCSV27-31 directly into $orf55_{TP901-1}$ of the lysogenic TP901-1 genome by homologous recombination, which was obtained by succeeding incubation steps at alternating temperatures as previously described (3, 38). The lysogenic strain containing TP901-1*erm* was verified by colony PCR for the presence of the *erm* gene in $orf55_{TP901-1}$ and the loss of the pGhost8-derived vector (results not shown). The phage TP901-1*erm* was induced from the lysogenic strain and purified by isopycnic centrifugation. According to previous observations (30), TP901-1*erm* was found to infect *L. lactis* 3107 with the same efficiency as TP901-1 wt (results not shown).

Generation and isolation of chimeric TP901-1/Tuc2009 phage. By use of the splicing-by-overlap-extension PCR technique (27), a fragment of recombinant phage DNA was amplified from purified TP901-1 and Tuc2009 DNA with the following oligonucleotides: F-CV007 (5′-CGCGGATCCATGCGGATGTCA ATAGTCAAGCCATTGTTG), R-CV035 (5′-CCTATTTCTATTAAGCTA CAAAAACATAGC), F-CV036 (5′-TGTAGCTTAATAGAAATAGGAGAA TAAAATG), R-CV037 (5′-CCCCTACTTTCTAATTCCGATAAAGTTTTAC), F-CV038 (5′-TCGGAATTAGAAAGTAGGG-GTTATGGAGG), and R-CV039 (5′-ACGCGTCGACAAATTTTCAGGACTAATACC) (the incorporated flanking BamHI and SalI sites are underlined). This PCR fragment contained the Tuc2009 gene $orf53_{2009}$ with the Shine-Dalgarno sequence inserted between $bppU_{TP901-1}$ and $orf50_{TP901-1}$, hence creating a 2.3-kbp recombinant phage sequence in which $orf53_{2009}$ was inserted in exchange for $bppL_{TP901-1}$ (Fig. 1 and Table 1). The recombinant phage fragment was inserted as a BamHI-SalI fragment into the vector pCI372, thereby producing pCSV71-8. Following the transformation of *L. lactis* MG1363 and subsequent plasmid purification, pCSV71-8 was finally introduced into the TP901-1 host strain *L. lactis* 3107 by transformation.

In order to obtain chimeric phages with $BppL_{TP901-1}$ exchanged for $ORF53_{2009}$, the TP901-1*erm* phage was propagated in the presence of the recombinant TP901-1/Tuc2009 fragment. *L. lactis* 3107 cells harboring pCSV71-8 were grown in GSB medium supplemented with 5 μg/ml chloramphenicol until early in the exponential phase (optical density at 600 nm [OD_{600}] of 0.1), at which stage TP901-1*erm* phages were added at a multiplicity of infection (MOI) of less than 0.02. The infected culture was wrapped in foil and incubated at room temperature (about 23°C) until complete lysis was obtained. Cellular debris was removed by centrifugation for 10 min at 7,500 × *g*, and the lysate was filtered by gravity at 4°C through a 0.45-μm-pore-size filter (Frisenette, Ebeltoft, Denmark). The titers of phages in the lysate were determined by plaque assay on TP901-1 host strain *L. lactis* 3107.

The lysate of TP901-1*erm* propagated in the presence of the recombinant phage fragment was analyzed for chimeric phages with an altered host range by testing for phages which could lysogenize and hence confer an Erm^r lysogenic conversion phenotype to the Tuc2009 host strain. Cells of *L. lactis* UC509.9 were grown to the exponential phase (OD_{600} of about 0.3), at which stage 3.5 ml cells was mixed with 1 ml phage lysate and calcium chloride to a final concentration of 5 mM. The mixture was incubated for 1 to 1.5 h at 30°C in order to allow for phage adsorption, DNA injection, and *erm* expression. The cells were subsequently collected by centrifugation for 10 min at 3,800 × *g*, resuspended in 0.5 ml GM17, added to 3 ml GM17 soft medium plus calcium chloride, and poured onto GM17 solid medium plus calcium chloride supplemented with 1 μg/ml erythromycin. Following an overnight incubation at 30°C, the plates were incubated for 3 days at room temperature. Colonies were inoculated in GM17 medium plus 1 μg/ml erythromycin and incubated at 30°C overnight. The supernatants were assayed for spontaneously released phages that could infect *L. lactis* UC509.9 in plaque assays.

Numerous unsuccessful experiments using the method described above as well as direct testing for TP901-1 wt-derived chimeric phages by plaque assays on *L. lactis* UC509.9 were carried out prior to the isolation of a single chimeric phage. The primary problem was to obtain a sufficiently high titer in the phage lysate containing the chimeric phages. Lysate titers were generally at the level of 10^7 to 10^9 PFU/ml, as is regularly observed for lytic propagation of TP901-1 on *L. lactis* 3107 (L. Brøndsted, unpublished data), and only once was a lysate with a titer of 6×10^{11} PFU/ml obtained.

Plaque, adsorption, and lysogenic conversion assays. Phage titers were determined by plaque assay on TP901-1 host strain *L. lactis* 3107 or Tuc2009 host strain *L. lactis* UC509.9 in GM17 medium supplemented with 5 mM calcium chloride and agarose (Invitrogen), as described previously by Lillehaug (32). Phage adsorption assays for *L. lactis* 3107 and *L. lactis* UC509.9 were performed as described previously by Garvey et al. (16). In brief, late-exponential-phase cells or GM17 medium was mixed with phages (approximately 10^5 PFU/ml) and calcium chloride. Following a 15-min incubation at room temperature, the mixture was cleared of cells by centrifugation, and the supernatant was assayed for

phages by plaque assay. The level of adsorption was calculated as 1 minus the ratio between phages (PFU/milliliter) in the cell supernatants and phages (PFU/milliliter) in the cell-free supernatant.

The frequency of lysogenic conversion (Erm^r) of *L. lactis* 3107 and *L. lactis* UC509.9 was determined by infecting 0.1 ml of early-exponential-phase cells (OD_{600} of 0.2 to 0.3) with 0.1 ml of phages (diluted in 10 mM calcium chloride–0.9% [wt/vol] sodium chloride) at an MOI of 0.5. Following a 20-min preincubation at room temperature, the mixture was added to GM17 soft medium plus calcium chloride and poured onto GM17 solid medium plus calcium chloride supplemented with 1 μg/ml erythromycin. After 3 days of incubation, the number of Erm^r CFU was determined, and the frequency of lysogenic conversion was calculated as the number of Erm^r CFU divided by the total number of added phages (PFU).

SDS-PAGE and Western blotting. Protein profiles of the phages were determined with approximately 10^{10} PFU of purified and denatured phage particles. The proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under reducing conditions with the NuPAGE system in 10% Novex Bis-Tris polyacrylamide precast gels and MES (morpholineethanesulfonic acid) SDS running buffer (Invitrogen, Carlsbad, Calif.) as previously described (47). Following electrophoresis, the protein bands were either silver stained with the SilverQuest Silver Staining kit (Invitrogen) or electroblotted onto a polyvinylidene difluoride membrane. A Western blot with polyclonal antibodies raised in rabbits against $ORF53_{2009}$ and a secondary labeled anti-rabbit antibody was carried out as previously described (47).

Transmission electron microscopy. Purified phage preparations were dialyzed against SM buffer (100 mM sodium chloride, 10 mM magnesium sulfate, 50 mM Tris [pH 7.5], 0.01% [wt/vol] gelatin), and a carbon film was floated from a mica sheet into the dialyzed phage suspension and incubated for 10 min. The film was subsequently rinsed in demineralized water and stained for 30 s with 2% (wt/vol) uranyl acetate (Agar Scientific, Stansted, United Kingdom). A 400-mesh copper grid (Agar Scientific) was used to pick up the carbon film, and the phages were examined with a transmission electron microscope (Tecnai 10; FEI Eindhoven, The Netherlands) at an acceleration voltage of 80 kV. Micrographs were taken with a MegaView II charge-coupled-device camera (SIS, Münster, Germany).

Nucleotide sequence accession number. A sequence of the TP901-1C late promoter region has been deposited in the GenBank database under accession no. DQ093874.

RESULTS

Comparison of TP901-1 and Tuc2009 tail proteins. In order to identify potential candidates for the antireceptors of TP901-1 and Tuc2009, the tail proteins of the two phages were compared. TP901-1 and Tuc2009 have different host ranges, and their antireceptor proteins were therefore expected to show limited sequence similarity. Tail proteins encoded from the major tail protein (MTP) gene to the neck passage structure (NPS) gene were compared by their amino acid sequences, and it was observed that the majority of these proteins displayed a high degree of identity (74 to 100% over the entire sequence) (Fig. 1). Tail fiber proteins have often been found to constitute antireceptors of *E. coli* phages (for a review, see reference 23), but the TP901-1 tail fiber protein $Tal_{TP901-1}$ was found to display 93% sequence identity with Tal_{2009} of Tuc2009, and it was therefore considered unlikely that the tail fiber proteins were determinants for the host range difference between these two phages. While no homologous protein of Tuc2009 $ORF52_{2009}$ was found among the TP901-1 tail proteins, TP901-1 $BppL_{TP901-1}$ and Tuc2009 $ORF53_{2009}$ showed a weak identity (20%) over the entire sequence and 50% identity over the first 61 N-terminal amino acids. Recently, we identified $BppL_{TP901-1}$ as the protein of the lower baseplate disk of TP901-1, and our results indicated that $BppL_{TP901-1}$ may be the TP901-1 antireceptor (47). $BppL_{TP901-1}$ and $ORF53_{2009}$ are almost equal in size (163 and 174 amino acids) and share some degree of similarity; moreover, the two proteins have approximately the same genomic position. It was therefore hypothesized that these proteins were the antireceptor proteins of TP901-1 and Tuc2009, respectively.

Generation and isolation of a chimeric TP901-1 phage with an altered host range. In order to determine whether $BppL_{TP901-1}$ and $ORF53_{2009}$ constitute the respective antireceptors of TP901-1 and Tuc2009, attempts were made to isolate a TP901-1 phage with the host range of Tuc2009 by exchanging $BppL_{TP901-1}$ with $ORF53_{2009}$. First, a recombinant phage fragment was created in which $orf53_{2009}$ and the associated Shine-Dalgarno sequence were inserted between the up- and downstream flanking sequences of $bppL_{TP901-1}$, thereby replacing $bppL_{TP901-1}$ with $orf53_{2009}$ (Fig. 1). The fragment was generated using splicing-by-overlap-extension PCR in order to preserve the original sequence composition, i.e., to avoid the insertion of additional restriction sites in the surrounding sequences of $orf53_{2009}$. The recombinant fragment was cloned into the vector pCI372, forming pCSV71-8, and introduced into the TP901-1 host strain *L. lactis* 3107. The relatively low-copy cloning vector pCI372 was used for this experiment because of previous difficulties with the transformation of *L. lactis* 3107 with several high-copy lactococcal vectors (37). It was reasoned that $bppL_{TP901-1}$ of the TP901-1 genome could be exchanged for $orf53_{2009}$ by homologous recombination upon TP901-1 infection of *L. lactis* 3107 harboring pCSV71-8. When infected at a low MOI, the resulting lysate would contain chimeric TP901-1 phages, both genotypic and phenotypic $orf53_{2009}$, and according to the proposed hypothesis, these phages were expected to have an altered host range. A TP901-1 derivative (TP901-1*erm*) containing an Erm^r marker was used as the parental phage for the generation of these chimeric phages in order to overcome potential problems with the lytic propagation of the chimeric phage, as previously noted for other such lactococcal phages (14, 44). Lysates generated in these experiments were consequently analyzed for phages that could confer the Erm^r lysogenic conversion phenotype to the Tuc2009 host strain *L. lactis* UC509.9. Infection of *L. lactis* UC509.9 with a total of 6×10^{12} PFU (determined on *L. lactis* 3107) from a lysate of TP901-1*erm* propagated on *L. lactis* 3107 harboring pCSV71-8 resulted in a single Erm^r colony, which was found to spontaneously release *L lactis* UC509.9 infectious phages when cultured in liquid media. The phage of this lysogenic strain was named TP901-1C.

Genomic analysis of the chimeric phage TP901-1C. The genome of the chimeric phage was isolated from purified TP901-1C phages, and the region of the TP901-1C lower baseplate gene was amplified and sequenced to determine if the isolated phage had obtained the $orf53_{2009}$ gene in exchange for $bppL_{TP901-1}$. The sequencing results confirmed that $orf53_{2009}$ was correctly inserted between $bppU_{TP901-1}$ and $orf50_{TP901-1}$ of TP901-1C. A single point mutation was discovered (T→C at position 33,664 of the Tuc2009 sequence) (GenBank accession no. NC_002703), but this mutation did not change the amino acid sequence, and it was therefore considered to be insignificant (results not shown).

The genome of TP901-1C was analyzed with restriction endonuclease digestion in order to verify that TP901-1C was a TP901-1*erm* derivative with an altered host range. Phage genomic DNA of TP901-1 wt, TP901-1*erm*, TP901-1C, and Tuc2009 was isolated from purified phage preparations, di-

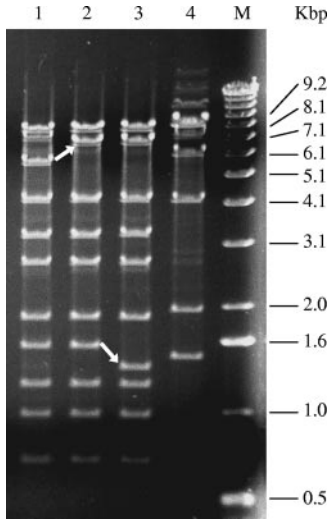

FIG. 2. EcoRV endonuclease restriction profiles of phage DNA genomes separated by agarose gel electrophoresis. Lane 1, TP901-1 wt; lane 2, TP901-1*erm*; lane 3, TP901-1C; lane 4, Tuc2009; M, 1-kb DNA ladder (Invitrogen). Sizes of double-stranded DNA fragments are indicated to the right of the gel. The upper arrow indicates the changed mobility of the fragment covering the inserted *erm* gene in TP901-1*erm* and TP901-1C. The lower arrow indicates the changed mobility of a TP901-1C fragment due to a secondary recombination.

gested with EcoRV, and analyzed by agarose gel electrophoresis (Fig. 2). Consistent with prior observations (30), the inserted 1.2-kbp *erm* gene in $orf55_{TP901\text{-}1}$ resulted in a changed mobility of a 5.7-kbp fragment, which was found to migrate together with a larger fragment in the TP901-1*erm* and TP901-1C profiles (Fig. 2, upper arrow). The restriction profiles clearly demonstrate that TP901-1C is a TP901-1*erm* derivative, as the TP901-1C profile was found to be very similar to the TP901-1*erm* profile and significantly different from the Tuc2009 profile. Equivalent EcoRI and PstI restriction profiles further confirmed these observations (results not shown). A single fragment of the TP901-1C EcoRV profile was, however, found to show a changed mobility compared to the TP901-1 wt and TP901-1*erm* profiles (Fig. 2, lower arrow). To investigate the reason for this altered single fragment mobility, the corresponding region of TP901-1C was amplified and sequenced. It was thus discovered that TP901-1C contained a 2.5-kbp region (GenBank accession no. DQ093874) that differed from the TP901-1 wt sequence. The impacts of this new sequence were the losses of $orf27_{TP901\text{-}1}$ and $orf28_{TP901\text{-}1}$, two small genes (97 and 47 codons, respectively) of unknown function in the middle transcript region of TP901-1 (7, 34). Furthermore, TP901-1C had acquired a new *alt* gene (*a*ctivator of *l*ate *t*ranscription), which showed 99% nucleotide identity and 100% amino acid identity with the corresponding *alt* gene from phage Tuc2009 (8, 42), and a changed −35 sequence of the late promoter (AC<u>A</u>TCA) with only a single nucleotide mismatch (underlined) with the corresponding region (AC<u>C</u>TCA) of phage Tuc2009. TP901-1C thus contained a late promoter region and regulator, which was highly similar to the corresponding region of phage Tuc2009 (Fig. 3). In order to investigate the origin of this unexpected new sequence, colony PCRs were performed with TP901-1C discriminative primers on *L. lactis* 3107 and *L. lactis* UC509.9. These PCRs revealed that the new sequence originated from *L. lactis* 3107, a result that was confirmed by sequencing of the fragment (1.2 kbp) amplified from this strain (results not shown). These results indicate that TP901-1C had obtained the new sequence by homologous recombination with the *L. lactis* 3107 genome.

Examination of TP901-1C adsorption and infection. The phage TP901-1C was found to form clearly visible plaques on the Tuc2009 host strain *L. lactis* UC509.9 and was readily propagated to a titer of 5×10^9 PFU/ml. In contrast, TP901-1C could not infect the TP901-1 wt host strain, as no plaques were

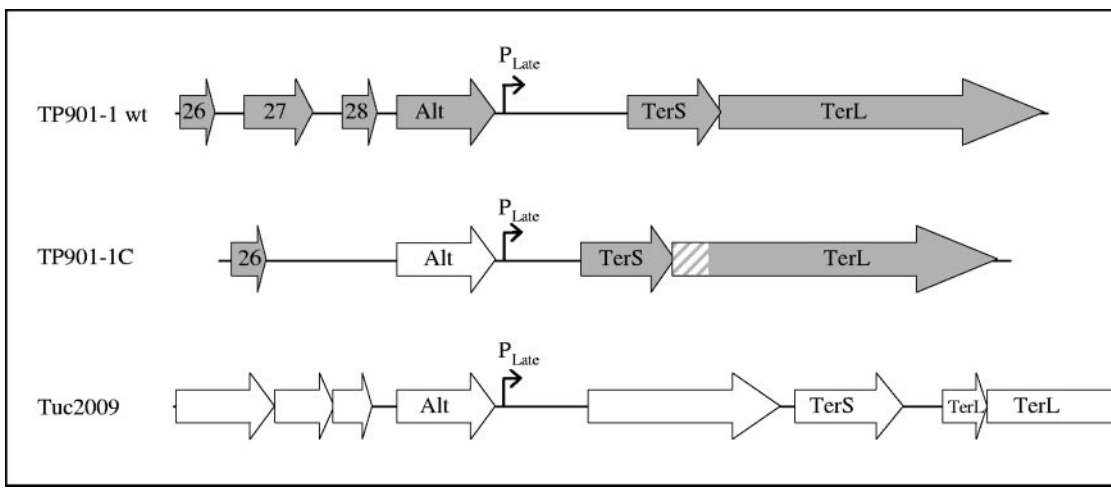

FIG. 3. The sequenced late promoter region of TP901-1C compared to the corresponding regions of TP901-1 wt and Tuc2009. The late promoters (P_{Late}) and relevant encoded proteins are indicated with protein names or the numbers of their encoding open reading frames. Identical amino acid sequences are shown with identical shades. $ORF27_{TP901\text{-}1}$ and $ORF28_{TP901\text{-}1}$ are absent from the TP901-1C sequence. The activators of late transcription (Alt) of TP901-1C and Tuc2009 display 100% amino acid identity. The late promoter sequence of TP901-1C is more closely related to that of Tuc2009 than to that of TP901-1 wt. The large-subunit terminase *terL* genes of TP901-1C and TP901-1 wt display a 10-nucleotide difference in the 5′ sequence.

TABLE 2. Infection, adsorption, and lysogenic conversion frequencies of TP901-1, TP901-1*erm*, Tuc2009, and TP901-1C

Phage	Infection[a]		Adsorption (%)[b]		Erm^r lysogenic conversion frequency (%)	
	3107[c]	UC509.9[d]	3107[c]	UC509.9[d]	3107[c]	UC509.9[d]
TP901-1 wt	+	−	93 ± 3	3 ± 5	ND[g]	ND
TP901-1*erm*	+	−	93 ± 2	8 ± 11[e]	0.002	$<10^{-7}$[f]
TP901-1C	−	+	16 ± 16	88 ± 3	$<10^{-7}$[f]	0.01
Tuc2009	−	+	8 ± 12	81 ± 10	ND	$<10^{-7}$[f]

[a] Infection ability determined by plaque assays. +, positive; −, negative.
[b] Means of three independent experiments and standard deviations.
[c] *L. lactis* 3107.
[d] *L. lactis* UC509.9.
[e] Means of two experiments.
[f] Outside the limits of the assay.
[g] ND, not determined.

observed in plaque assays with TP901-1C and *L. lactis* 3107 (Table 2). It was therefore concluded that TP901-1C has an altered host range compared to that of the parental TP901-1 phage.

In order to test whether the inability of TP901-1C to infect *L. lactis* 3107 was due to reduced phage adsorption or the blocking of later steps in the infection process, an adsorption assay was carried out. The results of this assay (Table 2) showed that TP901-1C displayed approximately the same adsorption characteristics as Tuc2009, i.e., a high level of adsorption to *L. lactis* UC509.9 but very limited adsorption to *L. lactis* 3107. These data strongly indicate that the failure of TP901-1C to infect *L. lactis* 3107 was due to its inability to adsorb to the TP901-1 host strain.

A lysogenic conversion assay was carried out to analyze the frequency of Erm^r lysogenic conversion and hence the infection efficiency of TP901-1C in comparison to that of the parental phage, TP901-1*erm* (Table 2). The obtained results revealed that the TP901-1C-mediated lysogenic conversion of *L. lactis* UC509.9 was as efficient as the TP901-1*erm*-mediated lysogenic conversion of *L. lactis* 3107, thus indicating that TP901-1C can infect *L. lactis* UC509.9 as efficiently as TP901-1*erm* can infect *L. lactis* 3107. The frequency of Erm^r lysogenic conversion by TP901-1*erm* obtained in this assay was approximately 10^3 times lower than that previously observed (30). Given that the result was confirmed by independent experiments (results not shown), we presume that the large difference in frequency is caused by different experimental conditions, e.g., the preincubation time and MOI value.

TP901-1C baseplate proteins. In order to verify that TP901-1C was a chimeric TP901-1 derivative, the structural proteins of TP901-1 wt, TP901-1*erm*, TP901-1C, and Tuc2009 were separated and evaluated by SDS-PAGE. A comparison of the protein profiles (Fig. 4A) revealed that the profiles of TP901-1 wt and TP901-1*erm* were identical, while the profile of TP901-1C diverged from these phages by the size of the smallest protein band (Fig. 4A, lower arrow). The smallest protein bands of TP901-1 wt and TP901-1*erm* were estimated to be approximately 17 kDa, while the corresponding band of TP901-1C was estimated to be approximately 19 kDa and was found to show the same size as a protein band in the Tuc2009 profile. This correlates well with the predicted 17.1- and 18.9-kDa molecular masses of $BppL_{TP901\text{-}1}$ and $ORF53_{2009}$, respectively (7, 42). Because the remaining protein bands of the TP901-1C profile, including the 35-kDa upper baseplate protein $BppU_{TP901\text{-}1}$ (Fig. 4A, upper arrow), were found to be indistinguishable from the TP901-1 wt and TP901-1*erm* profiles, the SDS-PAGE analysis revealed that TP901-1C is a TP901-1 derivative with a different lower baseplate protein.

To determine if the distinctive 19-kDa protein band of TP901-1C was in fact $ORF53_{2009}$, the protein profiles of TP901-1 wt, TP901-1*erm*, TP901-1C, and Tuc2009 were analyzed by Western blot using polyclonal antibodies raised against $ORF53_{2009}$. As illustrated in Fig. 4B, the antibodies reacted strongly with the 19-kDa $ORF53_{2009}$ band of Tuc2009 and less intensively with the 23-kDa major tail protein ($MTP_{TP901\text{-}1}$) and the 17-kDa $BppL_{TP901\text{-}1}$ of both TP901-1 wt and TP901-1*erm*. A strong antibody reaction to the distinctive 19-kDa band of TP901-1C was furthermore observed, and since no secondary reaction was observed with a band of 17 kDa from TP901-1C, the chimeric phage was concluded to contain the protein $ORF53_{2009}$ in exchange for $BppL_{TP901\text{-}1}$.

TP901-1C morphology. We have previously shown that $BppL_{TP901\text{-}1}$ constitutes the TP901-1 lower baseplate disk (47). To investigate if the exchange of $BppL_{TP901\text{-}1}$ for $ORF53_{2009}$ had caused any morphological changes in the TP901-1C baseplate, the purified phages of TP901-1 wt, TP901-1*erm*, and TP901-1C were examined and compared by transmission electron microscopy (Fig. 5). The baseplate of TP901-1 wt consists of two well-defined disks (Fig. 5A) (47), and the electron micrographs of TP901-1*erm* revealed that the inserted *erm* gene altered neither the baseplate nor the overall virion morphology, as TP901-1*erm* was found to be indistinguishable from TP901-1 wt (Fig. 5B). In contrast to this, the baseplate of TP901-1C was found to be clearly different (Fig. 5C), as the lower part of the chimeric baseplate looked like small hanging droplets rather than a defined disk. Moreover, the diameter of this lower part was found to be more narrow (23 ± 3 nm [n = 38]) than the lower baseplate disk from TP901-1 wt (28 ± 3 nm) (47). The overall appearance of the TP901-1C baseplate was estimated as an intermediate between the TP901-1 wt and

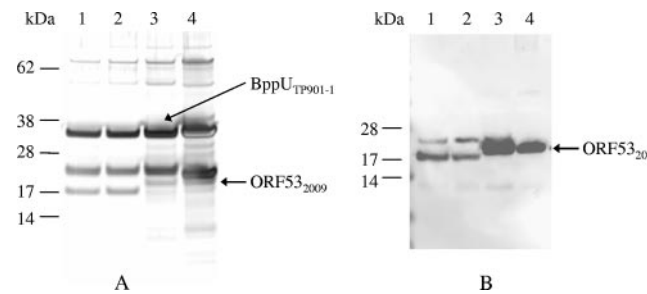

FIG. 4. (A) Silver-stained 10% SDS-PAGE gel with structural phage protein profiles. Lane 1, TP901-1 wt; lane 2, TP901-1*erm*; lane 3, TP901-1C; lane 4, Tuc2009. Molecular masses are indicated to the left of the gel. The upper arrow indicates the 35-kDa upper baseplate protein $BppU_{TP901\text{-}1}$ of TP901-1 wt, TP901-1*erm*, and TP901-1C. The lower arrow indicates the 19-kDa $ORF53_{2009}$ protein of TP901-1C and Tuc2009. (B) Western blot with polyclonal antibodies raised against $ORF53_{2009}$. Lane 1, TP901-1 wt; lane 2, TP901-1*erm*; lane 3, TP901-1C; lane 4, Tuc2009. Molecular masses are indicated to the left of the gel.

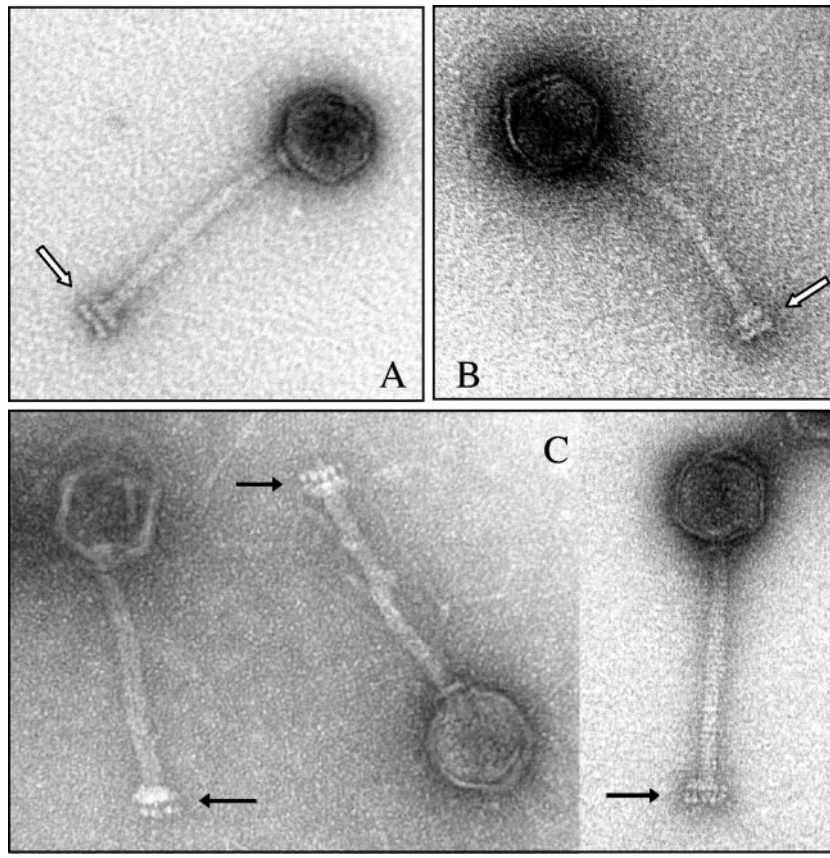

FIG. 5. Transmission electron micrographs of phages negatively stained with uranyl acetate. (A) TP901-1 wt; (B) TP901-1*erm*; (C) TP901-1C. White arrows point to the double-disk baseplate of TP901-1 and TP901-1*erm*. Black arrows point to the "hanging droplets" of the TP901-1C baseplate.

Tuc2009 baseplate morphology (47; S. Mc Grath et al., unpublished data). The remaining structures of TP901-1C were found to be identical to those of TP901-1 wt, supporting the previous observations of the lower baseplate being the only structural change of TP901-1C.

DISCUSSION

The infection processes of LAB phages and phages infecting gram-positive bacteria in general are poorly understood, and one of the primary reasons for this lack of insight is the limited knowledge of the host-interacting proteins and structures of these phages. This study describes the isolation and analysis of a chimeric *L. lactis* phage TP901-1 derivative with an altered host range.

The chimeric phage TP901-1C was constructed by homologous recombination between TP901-1*erm* (a TP901-1 phage carrying an *erm* gene) and a recombinant phage DNA fragment, wherein the $bppL_{TP901\text{-}1}$ gene was exchanged for $orf53_{2009}$ from the related phage Tuc2009. DNA sequencing, SDS-PAGE, and Western blot analyses of TP901-1C verified that the chimeric phage carried $ORF53_{2009}$ and not the TP901-1 lower baseplate protein $BppL_{TP901\text{-}1}$, while the remaining structural protein profile of TP901-1C was identical to the profiles of TP901-1 wt and TP901-1*erm*. A transmission electron microscopy examination showed that the chimeric phage has a baseplate structure different from the characteristic double disks of TP901-1. The morphology of the lower baseplate of TP901-1C was determined to be a more narrow structure with the appearance of small droplets hanging from the upper disk, while the remaining TP901-1C virion was found to be morphologically indistinguishable from those of TP901-1 wt and TP901-1*erm*. On the basis of these analyses, we conclude that TP901-1C is a chimeric TP901-1 phage in which $BppL_{TP901\text{-}1}$ is exchanged for $ORF53_{2009}$, and this exchange causes a morphological alteration of the TP901-1C baseplate.

TP901-1C was isolated as a prophage integrant of the lysogenic *L. lactis* UC509.9 strain. The chimeric phage had lost the ability to infect the TP901-1 host strain *L. lactis* 3107 while it acquired the ability to infect the Tuc2009 host strain *L. lactis* UC509.9. Furthermore, TP901-1C was readily propagated to sufficient titers ($>10^9$ PFU/ml) for phage purification by isopycnic centrifugation, and it is therefore estimated that TP901-1C and Tuc2009 infect *L. lactis* UC509.9 with approximately the same efficiency. Based on these results, we conclude that TP901-1C has an altered host range compared to that of the parental TP901-1 phage.

Restriction endonuclease digestion of the TP901-1C genome verified that the chimeric phage was a TP901-1*erm* derivative. However, the EcoRV profile and subsequent sequencing revealed that TP901-1C contains an unexpected non-

TP901-1 sequence region located approximately 16 kbp upstream of the exchanged baseplate gene. By sequencing of an *L. lactis* 3107 fragment, it was revealed that TP901-1C most likely had acquired this sequence by homologous recombination while propagated on *L. lactis* 3107 harboring pCSV71-8. This observation is in agreement with several previous reports of lactococcal phages of the P335 species frequently recombining with putative prophage sequences of their *L. lactis* host strains (4, 15, 36). The outcome of this secondary recombination was a changed late promoter region highly similar to the corresponding region of phage Tuc2009 and the loss of $orf27_{TP901\text{-}1}$ and $orf28_{TP901\text{-}1}$, two very small genes of unknown function.

The chimeric phage TP901-1C was isolated as a single phage with an altered host range from 6×10^{12} PFU; i.e., the phage was obtained with a frequency of approximately 10^{-13}. This frequency is several orders of magnitude lower than those reported from similar experiments with LAB phages (12, 14, 44). We believe this low frequency may be caused by the following factors. (i) The method used to isolate the chimeric phage in this study requires both a double-crossover recombination event and Erm^r lysogenic conversion, i.e., infection, lysogenization, and *erm* expression, an aspect supported by the finding of a 10^{-4} lysogenic conversion frequency of TP901-1C. (ii) The parental pCI372 vector used for the construction of the recombinant phage plasmid is not a high-copy vector like, e.g., the vector used in a related study by Duplessis and Moineau (12). (iii) *L. lactis* UC509.9 has been found to carry a restriction/modification system which decreases the efficiency of plating by 10^4-fold for unmodified phages (43).

It has previously been argued that the host specificity of phages involves more host-compatible features than the phage antireceptor (14, 44), and it would seem natural to suggest that the altered late promoter region of TP901-1C is a prerequisite for *L. lactis* UC509.9 infection. The chimeric phage was, however, isolated as a prophage integrant of a lysogenic *L. lactis* UC509.9 strain, and transcription of the lytic genes from the late promoter was therefore not required for TP901-1C infection, i.e., lysogenization of *L. lactis* UC509.9. The *erm* gene of TP901-1C is expressed constitutively from its own promoter (6), and the Erm^r phenotype of the TP901-1C lysogenic strain is therefore also independent of transcription from a phage promoter. This is in agreement with the results of the lysogenic conversion assay, which verified that TP901-1C efficiently lysogenizes *L. lactis* UC509.9. Recently, it was reported by Rakonjac et al. (40) that the acquirement of a new sequence adjacent to the *cosR* site in *L. lactis* phage c2 could overcome a host infection inhibition mechanism acting on the level of DNA injection or circularization by *cos*-end ligation. The corresponding *pac* site of TP901-1 is expected to be located in the vicinity of the late promoter (7), i.e., within the secondary recombination of TP901-1C. It is therefore possible that the secondary recombination of TP901-1C reflects a requirement for an altered *pac* site in order to overcome further inhibition mechanisms of *L. lactis* UC509.9. Nevertheless, neither the late promoter region nor the *pac* site is expected to have any effect on the initial host receptor interaction, and we therefore conclude that $BppL_{TP901\text{-}1}$ and $ORF53_{2009}$ are antireceptor proteins of TP901-1 and Tuc2009, respectively, and these proteins can promote adsorption to the host cells. Certainly, it cannot be excluded that other proteins participate in the adsorption and host receptor binding, but any such proteins are expected to play a minor host-specific role in this initial infection process, as the substitution of $BppL_{TP901\text{-}1}$ and $ORF53_{2009}$ was sufficient to completely alter the host adsorption of TP901-1C. The genomic comparison of TP901-1 and Tuc2009 showed that Tuc2009 carries an additional tail gene ($orf52_{2009}$) encoded between the upper baseplate protein homologue ($ORF51_{2009}$) and the antireceptor $ORF53_{2009}$ (Fig. 1). The genomic position of this gene indicates that $ORF52_{2009}$ participates in host infection, as previously suggested by Seegers et al. (42), but the present study shows that $ORF52_{2009}$ is in fact not required for TP901-1C infection of the Tuc2009 host strain *L. lactis* UC509.9. It is, however, possible that $ORF52_{2009}$ has a function in the host-interacting process of Tuc2009, a function that could possibly be structural stabilization/flexibilization of the Tuc2009 baseplate or host range extension/enhancement of the adsorption efficiency, as suggested for the dispensable long tail fibers of *E. coli* phage λ (25). Investigations have been initiated in order to explore these possible functions of $ORF52_{2009}$ in the infection process of Tuc2009. Moreover, it has been suggested that the TP901-1 tail fiber protein $Tal_{TP901\text{-}1}$ and the lytic active Tal_{2009} protein of Tuc2009 participate in the initial host infection processes by degradation of peptidoglycan to promote access to the host receptor or by assisting the DNA injection process analogous to the straight tail fiber of *E. coli* phage T5 (29, 47). The functions of $Tal_{TP901\text{-}1}$ and Tal_{2009} are, however, expected to be host unspecific, as these proteins share 93% amino acid identity (Fig. 1).

The results of the present study have identified antireceptor proteins of the temperate *L. lactis* phages TP901-1 and Tuc2009. This is the first identification of baseplate-located antireceptors from lactococcal phages and the first identification of antireceptor proteins of phages from the P335 species. To our knowledge, this is also the first time an exchange of such proteins has been shown to result in altered phage morphology. The results of the present study have prompted further investigations of the host-interacting mechanisms of TP901-1 and Tuc2009. $BppL_{Tp901\text{-}1}$ and $ORF53_{2009}$ will be employed to identify the respective host receptors, which are largely unknown for lactococcal phages of the P335 species. Of particular interest is whether these phages use a membrane protein receptor equivalent to those of phages of the c2 species (18) or bind to cell wall components as previously suggested for phages of the 936 species (13, 19, 46). The distal tail structures and proteins of TP901-1 and Tuc2009 are possibly the best characterized among LAB phages (29, 38, 47; Mc Grath et al., unpublished). It will therefore be possible to consider the functions of individual tail proteins in future host interaction studies of these phages, which will ultimately lead to the elucidation of infection mechanisms and hence a better understanding of the host interaction mechanisms of LAB phages and phages infecting gram-positive bacteria in general.

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