Bacteriophage Tuc2009 Encodes a Tail-Associated Cell Wall-Degrading Activity

John G. Kenny,[1] Stephen McGrath,[1] Gerald F. Fitzgerald,[1,2] and Douwe van Sinderen[1,2]*

National Food Biotechnology Centre and Department of Microbiology[1] and Alimentary Pharmabiotic Centre, Biosciences Institute,[2] National University of Ireland, Cork, Ireland

Tuc2009 is a P335-type member of the tailed-phage supergroup *Siphoviridae* and was originally identified as a resident prophage of the gram-positive bacterium *Lactococcus lactis* UC509. A Tuc2009 gene designated tal_{2009} which is located within the morphogenic module was shown to specify a lytic activity within the 3′ portion of its coding region. Comparative sequence analysis indicated that the cell wall-degrading part of Tal_{2009} is a member of the M37 protein family and that Tal_{2009} lacks a cell-binding domain, a finding supported by binding studies. Tal_{2009} appears to undergo self-mediated posttranslational processing in both *L. lactis* and *Escherichia coli*. Antibodies directed against a purified C-terminal portion of Tal_{2009} were used for immunoelectron microscopy, which showed that Tal_{2009} is located at the tail tip of Tuc2009. Antibody neutralization studies demonstrated that Tal_{2009}-directed antibodies inhibited the ability of phage to mediate host lysis by more than 100-fold. These data indicate that tal_{2009} encodes a tail-associated lysin involved in localized cell wall degradation, thus allowing the Tuc2009 DNA injection machinery access to the membrane of its bacterial host.

Lactic acid bacteria are economically important bacteria used in the production of fermented foods such as cheeses, yogurts, and sausages. Tuc2009 is a 38,347-bp lysogenic member of the P335 type of the *Siphoviridae* supergroup of non-contractile-tailed bacteriophages (GenBank accession no. NC_002703) and was originally identified in *Lactococcus lactis* subsp. *cremoris* UC509, a strain used in Cheddar cheese production, following mitomycin C induction (2, 42).

Muralytic enzymes or lysins degrade the peptidoglycan (PG) matrix and play essential roles for both phages and bacteria. "Autolysins" is the term used for lysins which are produced by bacteria and involved in cell division, while the term "endolysins" refers to lytic enzymes involved in phage release. Some bacteria also produce lysins which act as class III bacteriocins. Lysins fall into three categories, glycosidases, amidases, and endopeptidases, depending on the type of chemical bond they cleave within the PG. Glycosidases can be further subdivided into the muramidases, glucosaminidases, and transglycosylases (55). Progeny release for many double-stranded-DNA-tailed phages has been shown to employ a lysis system involving one or more holins in conjunction with an endolysin. The holins function by forming pores in the cytoplasmic membrane of the host, thereby abolishing membrane potential and allowing the endolysin to access the PG layer.

Lysins exhibit a modular design (16). While a portion (usually the N-terminal part in the case of endolysins) encodes bond cleavage, a second segment is involved in substrate binding. This is believed to help the enzymatic efficiency and specificity of such muralytic enzymes by locating the active motif directly at the site of the substrate and causing endolysins to lyse only those bacteria possessing both the specifically recognized binding region and the target bond of the cleaving domain. It is this specificity of target recognition that could make lysins attractive therapeutic agents. Indeed, studies have demonstrated the usefulness of lysins by specifically lysing streptococci which had colonized mice (38). The lysin is thus said to demonstrate independently functioning domains, as shown for the choline-binding motif of the majority of lysins of *Streptococcus pneumoniae* and its phages (16) and the endolysin of Tuc2009 (50). Furthermore, the level of homology between these modules from endolysins and autolysins is supportive of the modular theory of phage evolution, as it indicates that the genes encoding such enzymes have arisen as a result of genomic exchange and rearrangement (16).

While the cellular PG layer gives structural support to the bacterium, it also represents a formidable barrier across which the phage must transport its DNA during the infection process. Several proteins used by phages infecting gram-negative bacteria to perform this task of "hole punching" have been characterized (45). Phages T4, T7, PRD1, and φ6, all of which infect gram-negative hosts, have been shown to incorporate a lysozyme, two transglycosylases, and an endopeptidase, respectively, in the mature virion (9, 36, 37, 44). In addition, an endolysin was identified as a structural component of PRD1 (46). The entry-associated lysins of T4, T7, PRD1, and φ6 are located at the tail, within the phage head, in the internal membrane, and in the nucleocapsid, respectively. These structural positions appear to be optimal locations for the lysin to contact the PG layer given the distinctive methods of cell entry employed by each phage. In the cases of PRD1 and T7, mutations in the entry-associated lysins did not stop infection but merely delayed it. For gp16 of T7 this delay only applies under conditions in which the PG layer undergoes higher-than-normal levels of cross-linking.

The thickness of the PG layer in gram-negative bacteria is

* Corresponding author. Mailing address: National Food Biotechnology Centre and Department of Microbiology, National University of Ireland, Cork, Ireland. Phone: 353 21 490 1365. Fax: 353 21 490 3101. E-mail: d.vansinderen@ucc.ie.

TABLE 1. List of bacteriophage, bacterial strains, and plasmids used in this study

Phage, strain or plasmid	Relevant feature(s)	Source or reference
Phage		
Tuc2009	Isolated from *L. lactis* subsp. *cremoris* UC509 following induction	13
c2	Prolate-headed lytic phage	34
Strains		
E. coli		
M15	Host for pQE60 plasmids; contains pREP4, Kan^r	Qiagen
EC101	*E. coli* JM101 with chromosomally encoded *repA*	28
L. lactis		
UC509.9	Prophage-cured derivative of *L. lactis* subsp *cremoris*, host for Tuc2009	13
MG1614	Host for c2	19
MG1363	Plasmid-free derivative of NCDO712	19
NZ9800	NZ9700 derivative; Δ*nisA*	25
S. thermophilus		
CNRZ01205.3	Prophage-cured derivative of *S. thermophilus* CNRZ01205	18
S. aureus		
ATCC 14458	*S. aureus* subsp. *aureus* Rosenbach	ATCC^a
Plasmids		
pQE60	*E. coli* expression vector, Amp^r	Qiagen
pNZ8048	*L. lactis* expression vector, Cm^r	15
pTal$_{2009}$-1	pQE60 + *tal*$_{2009}$ of Tuc2009	This study
pTal$_{2009}$-2ΔN	pQE60 + 1,617 bp of 3′ end of *tal*$_{2009}$ of Tuc2009	This study
pTal$_{2009}$-3ΔN	pQE60 + 1,320 bp of 3′ end of *tal*$_{2009}$ of Tuc2009	This study
pTal$_{2009}$-4ΔN	pQE60 + 852 bp of 3′ end of *tal*$_{2009}$ of Tuc2009	This study
pTal$_{2009}$-5ΔN	pQE60 + 501 bp of 3′ end of *tal*$_{2009}$ of Tuc2009	This study
pTal$_{2009}$-6ΔN	pQE60 + 381 bp of 3′ end of *tal*$_{2009}$ of Tuc2009	This study
pTal$_{2009}$ΔC	pQE60 + 2,520 bp of 5′ end of *tal*$_{2009}$ of Tuc2009	This study
pTal$_{2009}$mut-2ΔN	pQE60 + 1,617 bp of 3′ end of *tal*$_{2009}$ of Tuc2009 with SOEing mutation	This study
pGhost8	Mutagenising plasmid of lactococci	33
pTal$_{2009}$-5ΔNtet	PTal$_{2009}$-5ΔN + *tetK* from pGhost8	This study
pLys	pQE60 + *lys* of Tuc2009	This study
pNZTal$_{2009}$-2ΔN	pNZ8048 + NcoI-HindIII region from pTal$_{2009}$-2ΔN	This study

^{a} ATCC, American Type Culture Collection.

much less than that of their gram-positive counterparts, with estimated values ranging between approximately 2.5 and 7.5 nm and 20 and 50 nm, respectively (6, 26). In both cases the PG is expected to limit the size of diffusible molecules to about 50 kDa (14). Logically one would therefore expect phages infecting gram-positive bacteria to be accordingly equipped to passage their DNA across this obstacle, since this requirement would be more stringent for phages for which the hurdle is greater, as observed for gp16 of T7 (37). For Tuc2009, as for any other tailed phage infecting gram-positive bacteria, one would expect any lysin involved at the initiation of infection to be tail associated, although to our knowledge no data have been published to support this supposition.

In this study we describe a structural component of Tuc2009, ORF50, hereafter referred to as Tal$_{2009}$ (for "tail-associated lysin"), which exhibits lytic activity within the C-terminal portion of the protein. This tail-associated lysin encoded by the bacteriophage Tuc2009 was shown to be located towards the end of the phage tail and to undergo autocatalytic posttranslational processing. Binding of antibodies (Abs) specific to the lysin reduced the ability of the phage to infect its host. To our knowledge this is the first time a protein with lytic activity has been found to be a structural component in a phage infecting a gram-positive bacterium.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophages, and growth media. Bacteriophages, bacterial strains, and plasmids used in this study are listed in Table 1. *L. lactis* strains were grown in GM17 (Oxoid) broth or agar (1.4%) supplemented with 0.5% glucose at 30°C, while *Escherichia coli* strains were cultivated in Luria-Bertani (LB) broth or agar (1.4%) at 37°C (47). Bacteriophages Tuc2009 and c2 were propagated on *L. lactis* subsp. *cremoris* hosts UC509.9 and MG1614, respectively. *E. coli* M15 cells containing pQE60 or derivatives thereof were grown in the presence of 100 μg of ampicillin ml^{-1} and 25 μg of kanamycin ml^{-1}. The pQE60 derivative containing the tetracycline resistance gene of pGhost8 was maintained in *E. coli* with LB broth containing tetracycline at a final concentration of 5 μg ml^{-1}.

Phage preparations. Phages were purified using CsCl density gradient centrifugation (47). Where indicated, Tuc2009 was further purified by means of isopycnic centrifugation using a sucrose gradient. The latter procedure was performed by loading CsCl-purified phage onto 20 to 70% sucrose in TBT (100 mM Tris-HCl [pH 7], 100 mM NaCl, 10 mM MgCl$_2$) gradients, which had been preformed in clear SW41 tubes with a Hoefer SG gradient maker. The phage band was harvested following centrifugation at 35,000 rpm for 1.5 h at 4°C in a SW41 rotor with a Beckman L-60 ultracentrifuge. The virus particles were then suspended in TBT and pelleted by a further centrifugation at 35,000 rpm for 1 h at 4°C. The resultant phage pellet was resuspended in 100 μl of TBT. Plaque assays were performed as described by Lillehaug (31).

Sequence analysis. Database searches and protein family (pfam) allocations were performed using BLASTN and BLASTP (1) and using conserved domain search programs, located at the following URL (http://www.ncbi.nlm.nih.gov/). Sequence alignments were performed using the Clustal alignment method and MEGALIGN 3.16 software from a DNASTAR 2002 version 5 software package.

TABLE 2. List of oligonucleotides used in the construction of plasmids

Oligonucleotide name	Oligonucleotide sequence[a]
Tal$_{2009}$-1F	5′ AAA <u>CCA TGG</u> GTA ATA TCT TAT TTT TAG ATA AG
Tal$_{2009}$-2F	5′ AAA <u>CCA TGG</u> GCA ATA TCT CTG ACC TTG
Tal$_{2009}$-3F	5′ AAA <u>CCA TGG</u> CAG ATT TTA TAA ATG CAG
Tal$_{2009}$-4F	5′ AAA <u>CCA TGG</u> TCT CTA AAC AAG CGG CG
Tal$_{2009}$-5F	5′ AAA <u>CCA TGG</u> CCT TAA ACG GAC ACC CTG AAC G
Tal$_{2009}$-6F	5′ AAA <u>CCA TGG</u> CAA GTG AAA TGG GTT GG
Tal$_{2009}$mut-2ΔNF	5′ GGG CGA AGC TCT CGC CGC G
Tal$_{2009}$mut-2ΔNR	5′ CGC GGC GAG AGC TTC GCC C
Tal$_{2009}$-R	5′ GGA <u>AGA TCT</u> AAA TTT GAT ATA ATC CCT TGG ATT C
Tal$_{2009}$-RΔC	5′ GGA <u>AGA TCT</u> CGC ATG CTT GAT GAC CGT G
Lys F	5′ AAA <u>CCA TGG</u> AAA GAT TAA TCA AAA AAT C
Lys R	5′ GGA <u>AGA TCT</u> ATA ATT TAG TGT TTG ACC AGC
TetKF	5′ GGA ATT C[CA TAT G]GC TTC ACA GAA ATT CTA GAA CA
TetKR	5′ GGA ATT C[CA TAT G]GT TAA TAC GTG AGC TCT GCG AGG C

[a] NcoI and BglII sites are underlined once and twice, respectively. NdeI sites are in brackets.

DNA manipulations and sequencing. PCR amplifications were carried out using an EXPAND long template PCR system (Roche) according to the manufacturer's instructions with a Gene Amp PCR system 2400 thermal cycler (Perkin-Elmer) and the primers described in Table 2. Similarly, restriction enzymes, shrimp alkaline phosphatase, and T4 DNA ligase were supplied by Roche and employed as recommended by the manufacturer. Oligonucleotides were manufactured by MWG (Ebersberg, Germany). Purification of plasmids from *E. coli* was performed using a Wizard Plus SV Miniprep kit (Promega). Plasmid DNA preparations from *L. lactis* were completed using the protocol of O'Sullivan and Klaenhammer (41). Sequence analysis was performed by MWG (Ebersberg, Germany).

Plasmid construction. Specific PCR-generated DNA fragments representing sections of *tal$_{2009}$* or the complete *lys* gene (2) from Tuc2009 were produced. NcoI and BglII sites were incorporated into the relevant synthetic oligonucleotides to insert these PCR products into the *E. coli* expression vector pQE60 (Table 1). The stop codon that defines the 3′ end of either *tal$_{2009}$* or *lys* was omitted from complementary oligonucleotides, allowing a translational fusion of the various Tal$_{2009}$ sections or the Lys protein with the six-His tag encoded by pQE60. In the case in which the *tetK* gene was inserted into pTal$_{2009}$-5ΔNtet, an NdeI restriction site was included at both ends of the *tetK* PCR product via incorporation of the NdeI recognition sequence into both oligonucleotides. Otherwise, they were identical to those used by Maguin et al. to amplify the *tetK* gene with pGhost8 as a template (33). For the construction of pTal$_{2009}$mut-2ΔN, suitable primers were designed and site-directed mutagenesis was carried out using the PCR-based SOEing technique (22). To check expression of *tal$_{2009}$* in *L. lactis*, the DNA fragment corresponding to *tal$_{2009}$-2ΔN* was restricted from pTal$_{2009}$-2ΔN by the use of NcoI and HindIII and the smallest fragment generated by this digestion was ligated into the similarly restricted pNZ8048 to produce pNZTal$_{2009}$-2ΔN. This allowed transfer of the *tal$_{2009}$-2ΔN* fragment fused to the six-His codons from pQE60 in pTal$_{2009}$-2ΔN to a position downstream of the nisin-inducible promoter of the lactococcal expression vector pNZ8048 (15). Electrotransformation of plasmid DNA into *E. coli* was performed as described by Sambrook et al. (47), while that of *L. lactis* NZ9800 was performed as described by Wells et al. (54). All DNA cloning steps were initially performed using *E. coli* as a host. The integrity of all clones was checked by restriction profiling and DNA sequencing.

Protein expression and purification. Overexpression of target proteins was achieved using the *E. coli* expression plasmid pQE60 essentially as recommended by Qiagen. Induction was accomplished over a 4-h period and was initiated using isopropyl-β-ᴅ-thiogalactopyranoside (IPTG) (Sigma) at a final concentration of 1 mM when the culture had reached an optical density at 600 nm of 0.35 to 0.4. Protein expression in *L. lactis* from pNZ8048 was produced as outlined by de Ruyter et al. (15). Cultures of *L. lactis* or *E. coli* (30 ml) were used to express proteins for zymogram assays and autocleavage studies. The cells were harvested by centrifugation in a Hermle Z-323 centrifuge at 4,000 rpm for 20 min. Lysis was carried out by resuspending the pellet in 1 ml of lysis buffer (50 mM NaH$_2$PO$_4$ [pH 8.0], 1 M NaCl, 30 mM imidazole) and performing cell disruption in a mini-beadbeater-8 (Biospec products) for 10 min at 4°C.

Whole cells, cellular debris, and insoluble proteins were cleared by centrifugation at 14,000 rpm for 20 min in an Eppendorf benchtop centrifuge. Protein purification of Tal$_{2009}$-5ΔN involved overexpressing the protein in 300 ml of LB broth. Cells were harvested in a Beckman J2-21 centrifuge at 4,000 rpm for 20 min. The pellet was resuspended in 10 ml of lysis buffer and disrupted in an alcohol ice bath by ultrasonication (Soniprep 150) with 30-s bursts at an amplitude of 10 μm followed by 15-s breaks. This sonication was continued for 10 min. Lysates were cleared as described above, and the overexpressed TalT-5ΔN was purified by immobilized-metal-affinity chromatography. Briefly, this involved passing the lysate through a column containing 4 ml of nickel-nitrilotriacetic acid matrix (Qiagen) which had been preequilibrated with 10 ml of the lysis buffer. Two 10-ml washes were carried out using wash buffer (50 mM NaH$_2$PO$_4$ [pH 8.0], 1 M NaCl, 30 mM imidazole, 0.5% Triton X-100, 5 mM β-mercaptoethanol). The protein was then eluted using 10 ml of the elution buffer (50 mM NaH$_2$PO$_4$ [pH 8.0], 1 M NaCl, 250 mM imidazole) in approximately 1-ml aliquots. Protein concentrations were determined using a Bio-Rad protein assay in conjunction with a bovine serum albumin standard curve.

SDS-PAGE and zymogram assays. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (27) by the use of a 4% stacking gel and a 12% separating gel. Renaturing SDS-PAGE and zymogram analysis were carried out as outlined by Lepeuple et al. (29) except that whole-cell substrates were produced according to Sheehan et al. (51). When *Micrococcus lysodeikticus* ATCC4698 cells were employed, however, these were supplied in the form of a lyophilized powder (Sigma). Cells were incorporated into the gels at a final concentration of 0.4% (wt/vol). Protein sizes were compared to that of a prestained protein marker (New England Biolabs).

Western blotting and immunological detection. Following SDS-PAGE, proteins were transferred onto polyvinylidine difluoride (PVDF) membranes (Immobilon-P; Millipore) with a 0.1 M CAPS (3-[cyclohexylamino]-1-propanesulfonic acid; pH 11)–10% methanol transfer buffer (Mini-protean II; Bio-Rad Laboratories). Tetra-His horseradish peroxidase-conjugated Abs (Qiagen) were used at a 1/1,000 dilution according to the manufacturer's instructions. Detection using polyclonal Abs involved washes (2 × 10 min) of the membranes in TBS (10 mM Tris-HCl [pH 7.5], 150 mM NaCl) followed by a 10-min wash in TBS-Tween-Triton (20 mM Tris-HCl [pH 7.5], 500 mM NaCl, 0.05% Tween 20, 0.2% Triton X-100). Membranes were then incubated for 1 h in blocking buffer (TBS, 5% skimmed milk powder, 0.1% Tween 20). The three washes were repeated, after which the primary Ab was allowed to interact with its antigen by incubating the membrane in blocking buffer with an appropriate dilution of the polyclonal rabbit serum. Following the repetition of the three wash steps, the secondary Ab, horse radish peroxidase-conjugated anti-rabbit donkey immunoglobulin G (IgG) (Amersham Biosciences), was incubated with the membrane in the same manner as described for the primary Ab. Further washes (4 × 10 min) in TBS-Tween-Triton were performed, and the antigen-Ab complexes were detected using an ECL Western blotting detection system (Amersham Biosciences) according to the manufacturer's instructions. Where Tetra-His-detecting Abs were employed, protein sizes were determined using a six-His protein marker (Qiagen). To monitor production of Tal$_{2009}$ in vivo, 100 ml of a Tuc2009 lysate was added to 500 ml of an exponentially growing culture of UC509.9 at an optical density at 600 nm of 0.4 to give a multiplicity of infection (MOI) of approximately 1. Following this, 30-ml samples were taken at 20-min intervals. The cells were harvested and lysed using a bead-beater as described above. Equal concentrations of these protein samples were separated by SDS-PAGE, analyzed by West-

ern blotting, and detected as described above with the polyclonal rabbit Abs directed against Tal_{2009}-5ΔN.

N-terminal sequencing. Specific protein bands of interest were transferred to PVDF membranes by Western blotting as described above and excised from the membrane following visualization with Coomassie blue R-250. These were N-terminally sequenced by Alta Biosciences, University of Birmingham, England, using Edman degradation.

Cell wall binding. Assays to determine whether Tal_{2009} exhibited any cell wall binding capabilities were performed essentially as described by Buist (8). Briefly, 250 μl of exponentially growing UC509.9 cells was resuspended in an equal volume of a cell-free lysate of *E. coli* M15 cells expressing Tal_{2009} from $pTal_{2009}$-1 and incubated for 30 min at 30°C. Cell-free lysates expressing *lys* (2) from pLys were similarly incubated with UC509.9 cells as a positive control. These mixtures were centrifuged, and the cell pellets and supernatants were collected. The cells were washed with 250 μl of 0.5× M17 and resuspended in 250 μl of denaturation buffer (3), while 100 μl of the supernatants was dialyzed against distilled water and dried by centrifugation under a vacuum prior to being dissolved in 50 μl of denaturation buffer. Lysin activity was detected by zymogram analysis as described above.

Ab production and neutralization studies. Polyclonal Abs against purified TalT-5ΔN were raised in rabbits by CN Biosciences (UK) Ltd. An initial immunization with the protein of interest and Freund's complete adjuvant was followed by five subsequent boost injections of TalT-5ΔN. A final serum sample was acquired 11 weeks after the initial immunization. Specific test and control Ab preparations for immunoelectron microscopy were developed as described by Johnsen et al. (24). The glycine elution procedure from Harlow and Lane (21) was performed to specifically obtain only those Abs which bound to Tal_{2009}-5ΔN with a high level of affinity.

To perform Ab neutralization studies, a 5% inoculum from a fresh overnight culture of UC509.9 was set up in 100 μl of double-strength GM17 and left at 30°C. Samples (2 μl) were taken at hourly intervals to count CFU ml⁻¹. CsCl-prepared phage (2 μl) was added to render an MOI of 0.02 for c2 and MG1614 and for Tuc2009 and UC509.9. The cells were made up to a final volume of 200 μl with rabbit serum, which had been dialyzed against TBT and filter sterilized, and 2 μl of 1 M $CaCl_2$. Further 2-μl samples were taken up to 9 h after the first inoculation. Cell counts at each time point were performed by diluting the cells in Ringers solution, spread plating suitable dilutions on GM17 agar, and incubating the plates at 30°C.

Electron microscopy. A total of 5 μl of CsCl-purified Tuc2009 (~10^{11} PFU ml⁻¹) was dried onto a Formvar-coated copper grid (300 mesh) and stained with 2% (wt/vol) uranyl acetate. Immunoelectron microscopy was performed by initial incubation of 5 μl of the purified phages with 5 μl of the purified Ab preparations for 1 h at room temperature. These preparations were then dropped onto the Formvar-coated grids and allowed to attach for 10 min before being dried by the use of blotting paper. A total of 5 μl of the goat anti-rabbit IgG gold conjugate (Sigma) (5-nm-diameter grains) diluted 1/100 per the manufacturer's instructions (~7.5 × 10^9 particles) was then added onto the grid for a further 1-h incubation at room temperature. The excess liquid was blotted clear, and the grids were fixed in 0.25% glutaraldehyde–0.1 M $NaPO_4$ (pH 7.5), washed in H_2O, and finally stained as described above. The samples were analyzed using a Hitachi H-7000 transmission electron microscope.

RESULTS

Sequence analysis of *orf50* of Tuc2009 shows homology to a lytic enzyme. Cell wall-degrading enzymes involved in phage infection during the DNA injection process have been observed in gram-negative bacteria. To determine whether such proteins are also present in phage infecting gram-positive bacteria, a homology search of the proteins encoded by the region that was assumed to include the structural module of Tuc2009 was performed to identify any gene with sequence similarity to known lysins. The Tuc2009 region encompassing this structural module was deduced from the gene maps of previous comparative phage genome analyses (12, 42). One protein, the product of *orf50*, designated here as Tal_{2009} showed significant similarity (45% identity) to Zoocin A, a known lysin from *Streptococcus equi* subsp. *zooepidemicus* (52). This homology was confined to the region spanning amino acids (aa) 810 to 904 of

Tal_{2009}, a protein with a predicted length of 906 aa, and residues 56 to 157 of the 285-aa Zoocin A. Conserved-domain analysis of Tal_{2009} denoted the presence of an M23/M37 pfam domain at this C-terminal component of the protein. For the members of this family, homology with Tal_{2009} is generally confined to the specific conserved residues that associate the proteins with this pfam. Members of the M23/M37 family include zinc metalloendopeptidases, some of which are well characterized, mainly due to their lytic properties against known pathogens or because of their roles as virulence factors (4, 20, 30, 40, 43). Furthermore, the sequence of Tal_{2009} displays 94 and 89% identity to the entire expected protein sequence of ORF47 of TP901-1 (AF304433.1) and the complete structural protein from ul36 (AF349457.1), respectively. The 79% identity exhibited between protein 44 of the *L. lactis* IL1403 prophage bIL285 (AF323668.1) and Tal_{2009} is confined to approximately 550 aa at the C-terminal portion of both proteins. Thus, it appears that this C-terminal portion encodes a lysin as a conserved structural component of certain lactococcal phages.

Bacteriophage Tuc2009 contains a structural component with cell wall-degrading activity which can be assigned to Tal_{2009}. To detect whether Tuc2009 phage particles possess a structural component with lytic activity, zymogram assays were performed to visualize lytic functionality in CsCl-purified phage. A weakly visible lytic activity was observed, corresponding in size to that expected for the protein product of tal_{2009} (~100 kDa). This result is not shown here due to the poor visibility of this lytic zone, which could be caused by weak enzymatic activity, low concentration, a low level of mobility, or refolding difficulties of this protein. To confirm the predicted lytic activity encoded by tal_{2009}, the complete gene and five progressively smaller portions of the gene were amplified by PCR and individually inserted into the vector pQE60 to generate six different pQE60 derivatives (Table 1). The fragments were chosen to more precisely determine the specific region responsible for lytic activity within the protein. These proteins were overproduced in *E. coli* M15, and lysates of each of these strains were assayed for lytic abilities by zymogram analysis (Fig. 1). All of the fragments of the tal_{2009} gene, with the exception of the smallest one, were shown to specify a lytic activity. These results located the functional domain of apparent PG-degradative activity to within 166 C-terminal aa of Tal_{2009}, in accordance with what was indicated by bioinformatic analysis. When the zymogram assay was used, Tal_{2009} was found to be active against cell walls of *L. lactis* UC509.9 and MG1363 but ineffective against those of *Staphylococcus aureus* ATCC14458, *M. lysodeikticus* ATCC4698 (Sigma), and *Streptococcus thermophilus* CNRZ01205.3 (results not shown).

Comparative sequence analysis had failed to detect any cell-binding domain in Tal_{2009}. To verify this observation a cell-binding assay was performed using the endolysin of Tuc2009 as a positive control. From work performed by Sheehan et al. (50) Lys, the Tuc2009 endolysin, is known to include a cell wall-recognizing component. As expected, Lys exhibited binding characteristics whereas Tal_{2009} did not appear to bind the cells of *L. lactis* UC509.9 (Fig. 2A).

Tal_{2009} undergoes autoproteolysis at a specific site. From the zymogram assays it appeared that the three largest fragments of Tal_{2009} were subjected to a specific cleavage event
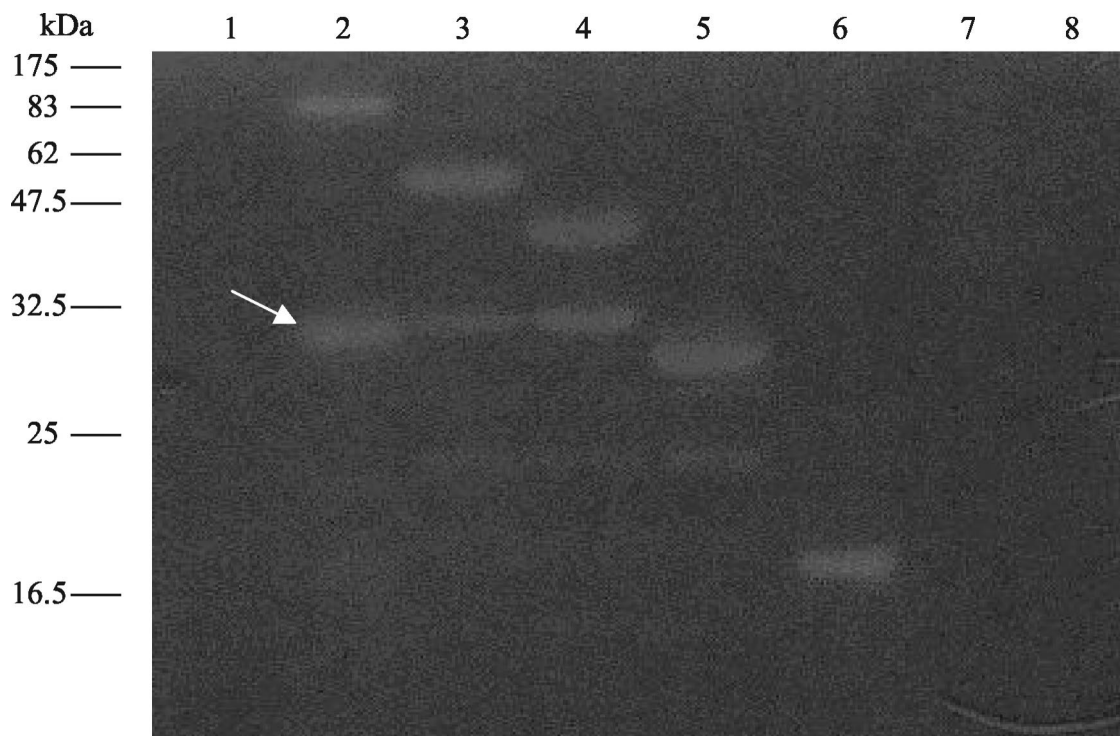
FIG. 1. Zymogram assay to determine lytic activity of Tal_{2009} in gels containing 0.4% (wt/vol) *L. lactis* UC509.9 autoclaved cells. Each well was loaded with *E. coli* M15 lysates with proteins expressed from the following plasmids: lane 1, pQE60; lane 2, $pTal_{2009}$-1; lane 3, $pTal_{2009}$-$2\Delta N$; lane 4, $pTal_{2009}$-$3\Delta N$; lane 5, $pTal_{2009}$-$4\Delta N$; lane 6, $pTal_{2009}$-$5\Delta N$; lane 7, $pTal_{2009}$-$6\Delta N$; lane 8, $pTal_{2009}\Delta C$. Molecular masses are indicated to the left of the gel, and a white arrow denotes the ~30-kDa processed proteins with lytic activity.

upon overexpression in *E. coli*, yielding a smaller lytic moiety of approximately 30 kDa (Fig. 1). To determine whether the observed processing was an *E. coli*-specific event, the second-largest fragment of tal_{2009}, including the six-His tag-encoding region, was restricted from pQE60 and ligated into pNZ8048. Upon electrotransformation into *L. lactis* NZ9000, production of the resultant protein was induced by nisin and lysates were analyzed for lytic activity by zymogram assay. As seen with *E. coli*, Tal_{2009} appeared to undergo processing because the zymogram assays displayed a smaller protein of ~30 kDa exhibiting lytic activity as well as a clear band of the expected size representing the entire protein being produced (data not shown). tal_{2009}-$2\Delta N$ was overexpressed in both *E. coli* and *L. lactis* and transferred to PVDF membranes by Western blotting before being subjected to N-terminal sequencing. In both cases (expression in *E. coli* and *L. lactis*), the ~30-kDa protein possessed the same N-terminal sequence, corresponding to cleavage of Tal_{2009} at a glycine-rich region GGSSG ↓ GG, where the arrow indicates the precise point where cleavage occurs. This finding is interesting, because the M37 protein family to which Tal_{2009} would seem to belong is known to include Gly-Gly endopeptidases. To ensure that this was indeed the cleavage site, gene SOEing was carried out which changed the naturally occurring GGSSGGG amino acid sequence to GRSSRRG by G-to-C transversions in the DNA sequence (mutated amino acid residues are underlined). The product of this gene was expressed in *E. coli*, and its lysis profile was compared to that of its nonmutated brethren. As

expected, only one zone of clearing was observed, corresponding to the unprocessed version of Tal_{2009} (Fig. 2B).

In an effort to discover whether autocatalysis was taking place, a deletion version of tal_{2009} was cloned into pQE60 to generate $pTal_{2009}\Delta C$. This deletion version of tal_{2009} is predicted to produce a truncated Tal_{2009} protein lacking the last C-terminal 66 aa and, as expected, was shown to be lytically inactive, failing to produce a zone of clearing in a zymogram assay (Fig. 1). The plasmid containing the smallest lytically active tal_{2009} fragment was modified by inserting the tetracycline resistance gene *tetK* into the NdeI restriction site of pQE60 to produce $pTal_{2009}$-$5\Delta Ntet$. This plasmid was then introduced into cells harboring plasmid $pTal_{2009}\Delta C$ and maintained by selecting for resistance to tetracycline. These two functional (Tal_{2009}-$5\Delta N$) and nonfunctional ($Tal_{2009}\Delta C$) proteins were then concomitantly expressed, and their interaction was analyzed by immunological detection. Interestingly, we found a discernible autoproteolytic mechanism whereby the larger inactive fragment was cleaved only in the presence of a lytically active segment of Tal_{2009}. This is visualized by the presence of a third fragment reacting to the anti-Tetra-His Abs (Fig. 3). The size of this fragment is commensurate to that which would arise if the $Tal_{2009}\Delta C$ protein were cleaved at the GGSSGGG region. The second protein fragment produced, corresponding to the N-terminal fraction of $Tal_{2009}\Delta C$, cannot be observed, since the Abs only bind to the C-terminal His-tagged portion of the Tal_{2009} proteins.
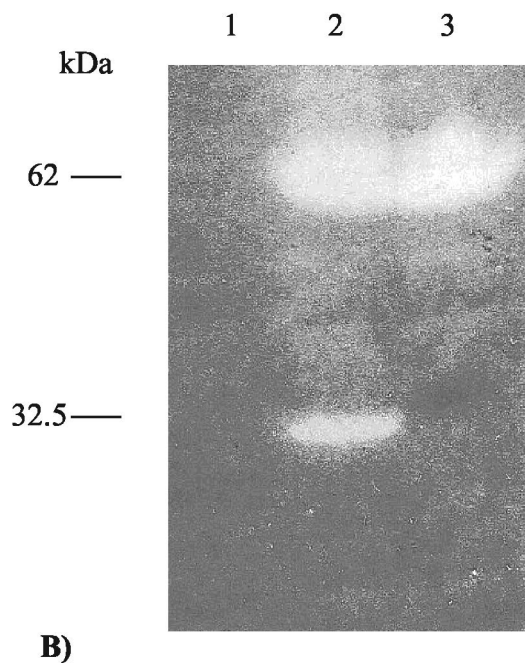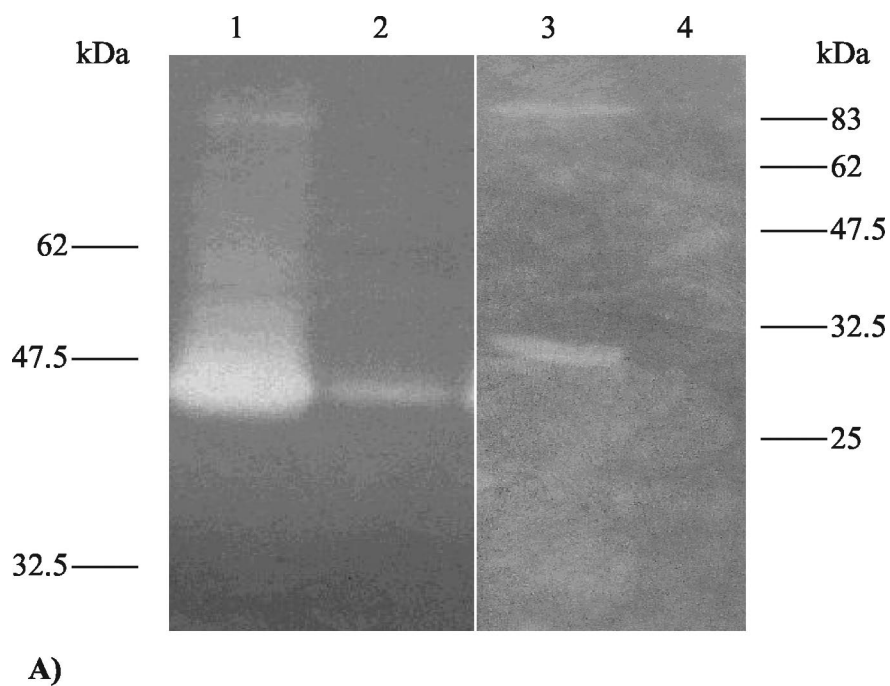
FIG. 2. (A) Zymogram assay to investigate any possible cell binding capabilities of Tal_{2009}. Lanes 1 and 2 show a zone of lytic activity corresponding to the Tuc2009 endolysin in the supernatant and cell pellet, respectively, following incubation with 509.9 cells. Lane 3 shows that Tal_{2009} is in the supernatant, while lane 4 shows Tal_{2009} to be absent from the 509.9 cell pellet. The gels contained 0.4% (wt/vol) *L. lactis* UC509.9 autoclaved cells. Molecular masses are indicated. (B) Zymogram analysis of the *E. coli* M15 lysates containing $pTal_{2009}$-2ΔN or the mutant $pTal_{2009}$mut-2ΔN in gels containing 0.4% (wt/vol) *L. lactis* UC509.9 autoclaved cells. Lane 1 contains the negative control pQE60, and lanes 2 and 3 contain $pTal_{2009}$-2ΔN and $pTal_{2009}$mut-2ΔN, respectively. Molecular masses are indicated to the left of the gel.
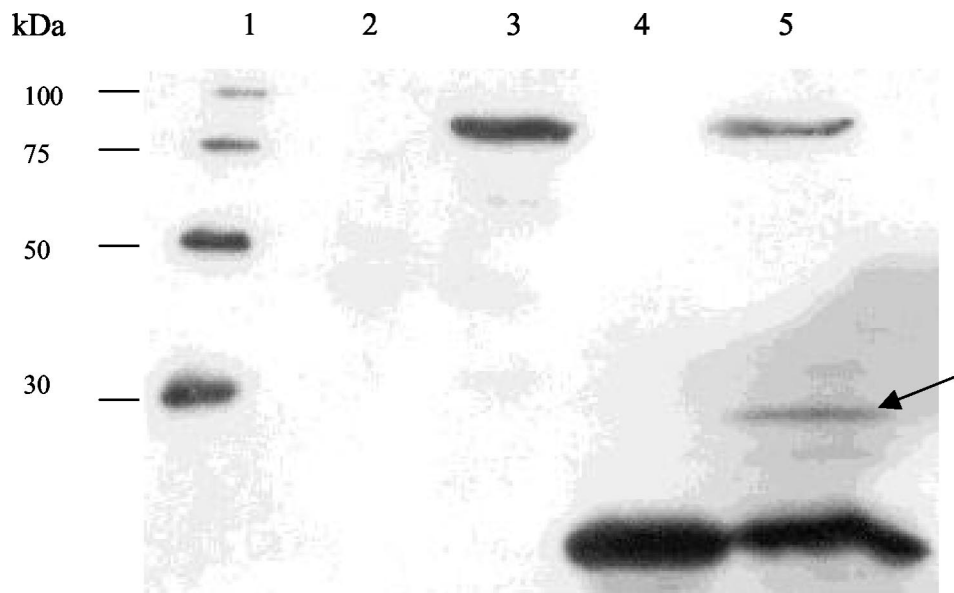
FIG. 3. Western blot to detect possible interaction between Tal$_{2009}$-Δ5N and Tal$_{2009}$ΔC. The negative control is in lane 2, and the lysates of *E. coli* cultures overexpressing protein from pTal$_{2009}$ΔC and pTal$_{2009}$-5ΔNtet are in lanes 3 and 4, respectively. Lysates resulting from concomitant induction of expression from pTal$_{2009}$-5ΔNtet and pTal$_{2009}$ΔC can be observed in lane 5, with a band of ~30 kDa denoted by an arrow. Lane 1 was loaded with a six-His protein ladder which reacts to the commercial Abs, and the molecular masses are indicated.

Identification of posttranslational processing of Tal$_{2009}$ in vivo and its incorporation into the mature phage virion. To elucidate the actual expression of Tal$_{2009}$ in vivo, its production was monitored using polyclonal Abs raised against the purified C-terminal moiety of Tal$_{2009}$ (Tal$_{2009}$-5ΔN). As detailed in Materials and Methods, 30-ml samples of a Tuc2009-infected culture were taken at intervals of 20 min for an hour and the harvested cells were disrupted using glass beads. The reaction to the Abs showed the production of two bands, which tally in size to the lytic activities seen upon overexpression of Tal$_{2009}$ in *E. coli* and *L. lactis* (Fig. 4A). These bands were first detected at 20 min following infection. To show that Tal$_{2009}$ in its entirety is assimilated into the mature phage, Tuc2009 isolated by CsCl gradient was immunologically analyzed. In lanes containing ~10^9 PFU of Tuc2009 phage particles, a single band was detected of the size expected for the full structural protein (Fig. 4B) and equal to that of the higher-molecular-weight band viewed upon zymogram analysis of the whole phage (results not shown). Determination of the N-terminal sequences of a number of structural proteins of Tuc2009 has indicated that unprocessed Tal$_{2009}$ is indeed part of the phage structure and that an approximately 60-kDa portion of it inclusive of the N-terminal end is also incorporated into the mature virion (J. Seegers, D. van Sinderen, and G. F. Fitzgerald, unpublished results).

Evidence for the localization of Tal$_{2009}$ within the tail of Tuc2009 and for its involvement during infection. To determine the importance of Tal$_{2009}$ in the phage multiplication process it was decided to perform an Ab neutralization experiment. When a range of MOI values from 0.02 to 20 was used, we observed a >2 log difference in viable counts between UC509.9 cells grown in the presence of terminal-bleed serum from the Tal$_{2009}$-5ΔN-immunized rabbits and those grown in the presence of the preimmunization-bleed serum (Fig. 5A).

No effect was seen on c2 infection of MG1614 (Fig. 5B). In addition, plaque assays were performed and PFU reductions of approximately 100-fold were observed for Tuc2009 compared to the results seen with a control (data not shown). c2 shows no homology to Tal$_{2009}$ from Tuc2009. Since the only difference between these two UC509.9 cultures is the presence of Abs against Tal$_{2009}$, these data suggest that Tal$_{2009}$ is involved in Tuc2009 infection, although binding of anti-Tal$_{2009}$ Abs may interfere with adjacent Tuc2009 structural proteins. To deduce the location of Tal$_{2009}$ (including the C terminus) within Tuc2009 particles, immunoelectron microscopy was undertaken. The lytic module was found to be situated towards the end of the tail (Fig. 6). The control (described in the Materials and Methods section above) did not react with Tuc2009, thereby indicating a lack of anti-Tal$_{2009}$ Abs in that preparation. Neither control nor test Ab preparations allowed the gold-conjugated anti-rabbit Abs to bind to c2 phage (data not shown). This is supportive of our findings obtained by homology searches and neutralization studies that c2 does not encode a homologue of Tal$_{2009}$. The fact that secondary Abs did bind to the tail of Tuc2009 indicated the presence of Tal$_{2009}$ at this location within the virion structure.

DISCUSSION

An increasing prevalence of multiple-drug-resistant pathogens has recently prompted interest in the methods employed by bacteriophage to lyse the host cell (32). Thus, work has been performed to determine the efficacy of lysins in killing colonizing pathogenic bacteria (38). In itself, this is an extension of bacteriophage therapy, a field undergoing a rejuvenation following a period spent loitering in the doldrums of Westernised medicine (35). One phage-encoded lysin has even been employed in the development of an in-field detection system for
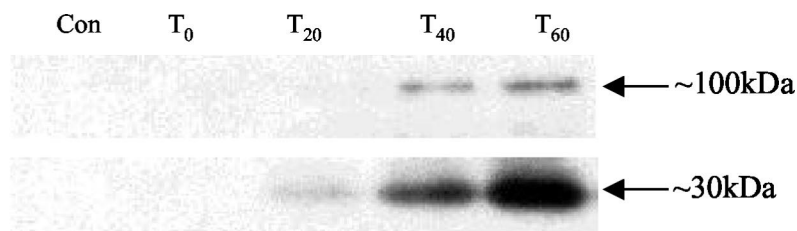
A)
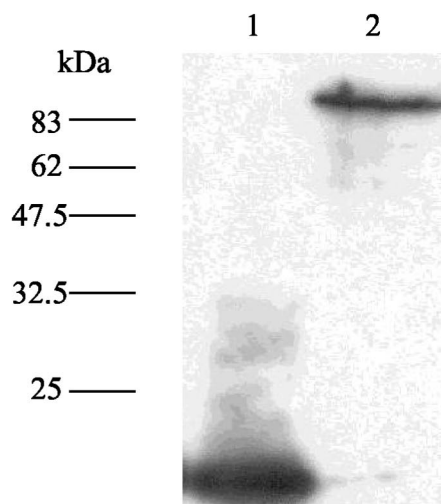
B)

FIG. 4. (A) Time point analysis of Tal_{2009} production in vivo including a lysate of uninfected UC509.9 cells to act as a negative control. The band sizes are indicated, with the lower band appearing slightly stronger than the larger band at 20 min (T_{20}). (B) Western blots of the mature Tuc2009 virion exposed a single band analogous in size to the full Tal_{2009} protein. Tuc2009 is in the second lane, while a lysate containing Tal_{2009}-5ΔN was run in lane 1 as a positive control. Molecular masses are indicated to the left of the gel.

Bacillus anthracis (49). Much work has been performed in addition to elucidate the mechanisms by which phages penetrate the PG layer upon DNA injection during the infection of gram-negative bacteria, while to our knowledge no data on this topic is available for gram-positive bacteria. One would expect PG to be a greater barrier to phages infecting gram-positive bacteria, given that the thickness of this component of the gram-positive cell envelope is significantly greater than that of the gram-negative equivalent.

Using zymogram assays we identified a lytically active protein component of Tuc2009 particles of ~100 kDa in size and identified the gene encoding this structural protein, Tal_{2009}. Bioinformatic analysis denoted that the C-terminal portion of Tal_{2009} is a member of the M23/M37 pfam, which includes

endopeptidases that target the interpeptide bridge of the PG layer. High levels of overall identity were recorded between Tal_{2009} and proteins of other phages infecting *L. lactis*. For the bacteriocin-like members of the M37 pfam, the similarities to Tal_{2009} and its closest phage-derived homologues are confined to those regions believed to be involved in enzymatic activity. Deletion analysis of Tal_{2009} did indeed show that the enzymatic activity is encoded by the 3′ portion of tal_{2009}. This agrees with results from the site-directed mutagenesis of LasA from *Pseudomonas aeruginosa* (20), which demonstrated that a His residue (underlined) within the conserved sequence VTG-PHLHF and also present in Tal_{2009} is essential for LasA activity. Upon expression of tal_{2009} in both *E. coli* and *L. lactis*, a strong and specific display of processing was exhibited which
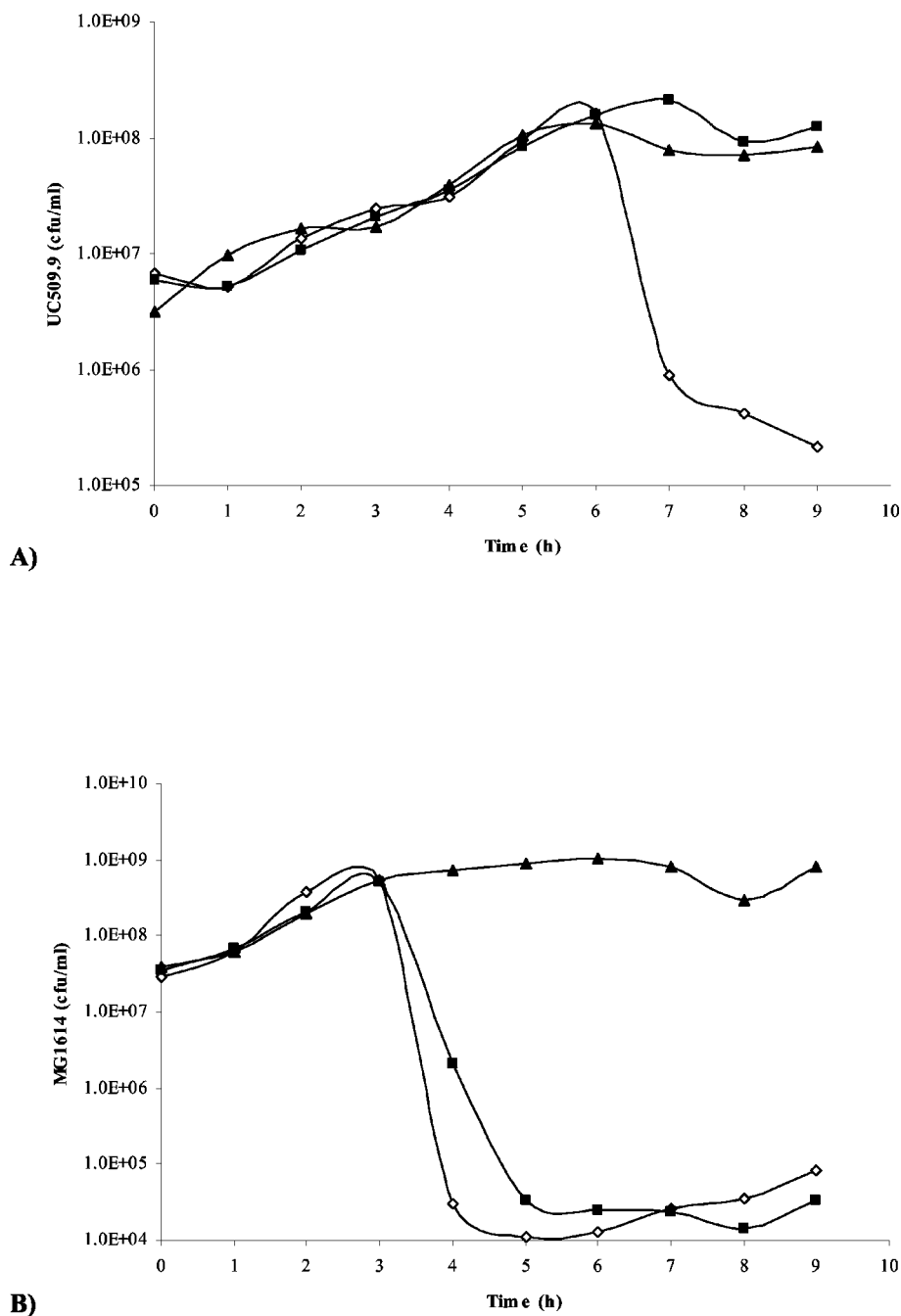
FIG. 5. (A) Graph of numbers of viable *L. lactis* UC509.9 cells versus time (hours). This graph is representative of results for a number of MOI values and times of addition of Tuc2009. (B) Graph of numbers of viable *L. lactis* MG1614 cells versus time (hours) upon infection with c2 phage at an MOI of 0.02. In both cases, cultures without phage, with phage and preimmunization bleed, and with phage and bleeds postimmunization with Tal_{2009}-5ΔN are denoted as closed triangles, open diamonds, and closed boxes, respectively.

gave rise to a secondary lytic zone approximately 30 kDa in size. Processing of phage structural proteins is a common phenomenon (10). N-terminal sequencing of the ~30-kDa Tal_{2009} fragment showed that cleavage occurred at a glycine (Gly)-rich region in both *E. coli* and *L. lactis*. In the case of the phage-encoded homologues of Tal_{2009} there are as many as eight glycine residues in a region of 10 aa. Glycine-rich regions within protein structures can act as flexible linkers between protein domains. It is possible, therefore, that this region in Tal_{2009} reflects a requirement of the phage to achieve mobility of the lytically active domain to effect hole formation in the PG layer while remaining incorporated in the phage structure. Interestingly, Gly-Gly regions are among the substrates targeted by members of the M23/M37 pfam, raising the possibility that this processing of Tal_{2009} is self-mediated. This was proven to be the case when a tertiary band of the expected size was
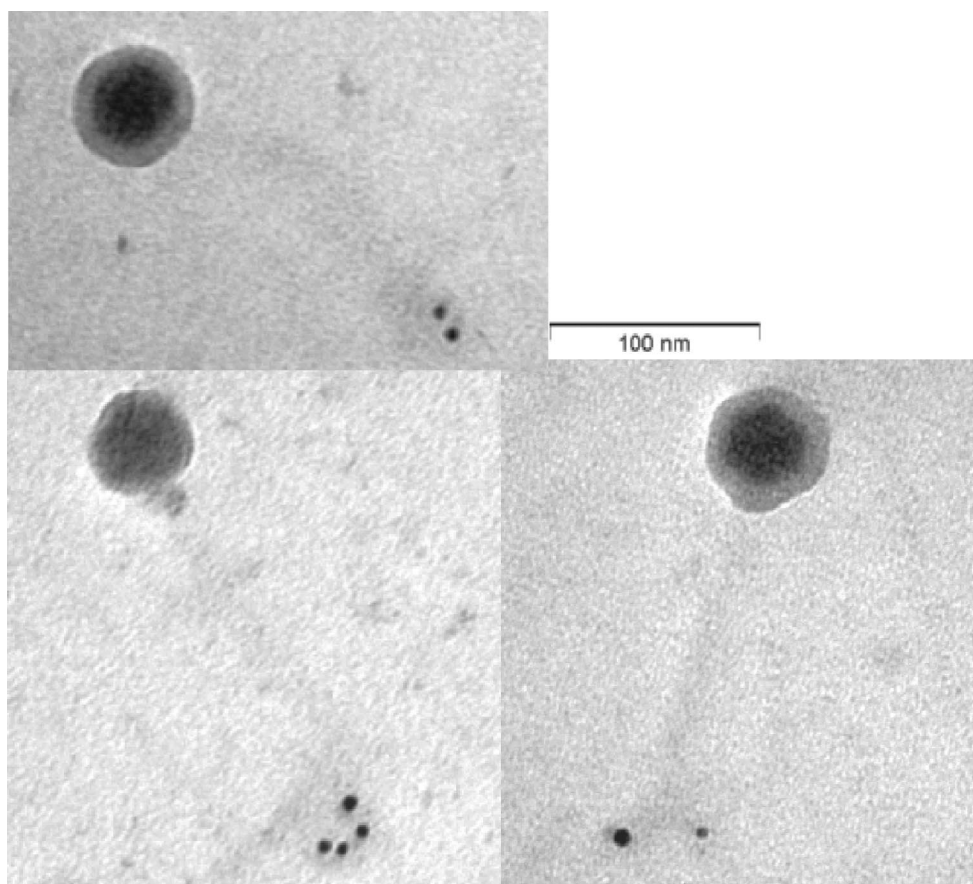
FIG. 6. Electron micrographs of Tuc2009 particles following incubation with rabbit anti-Tal$_{2009}$-5Δ N Abs and gold (5-nm-diameter grains)-conjugated goat anti-rabbit IgG (magnification, ×200,000).

produced upon coexpression of an inactive fragment of Tal$_{2009}$ (Tal$_{2009}$ΔC) with the smallest functional segment (Tal$_{2009}$-5ΔN).

One major problem exists with marrying the theories of Tal$_{2009}$ autocatalysis and lysis in that Tal$_{2009}$ recognizes a Gly-rich region for autocatalytic cleavage but does not lyse *S. aureus* cells which contain an interpeptide bridge in the PG layer that encompasses a stretch of five glycines, while it is instead capable of lysis of *L. lactis* cells where no such Gly residues exist within their cell walls. Members of the M23/M37 pfam cut the interpeptide bridge between different amino acid residues (5, 30, 53). The limited spectrum of Tuc2009 could be partially explained by the blinkered lysis spectrum of Tal$_{2009}$. Indeed, Rydman and Bamford (45) hypothesized that the incorporation of two lytic enzymes in the structure of PRD1 allowed for a broader host spectrum. There is virtually no homology between Tal$_{2009}$ and LasA or β-lp except at the residues that group all three in the same pfam. One could also speculate that Tal$_{2009}$ does recognize Gly-Gly in cell walls of *S. aureus* but that this bond is only weakly processed or perhaps that the flexibility of the Gly-rich region of Tal$_{2009}$ predisposes it to autocleavage via increased exposure.

Given that Tal$_{2009}$ in its entirety is included in the mature Tuc2009 phage, one would expect that too much processing to yield the smaller fragments could hinder virion construction or indeed functionality of the mature phage. In addition, immunological detection showed that the C terminus of Tal$_{2009}$ is only present in mature Tuc2009 as part of the full protein (Fig. 4B). It is entirely possible, indeed probable, that the processing of Tal$_{2009}$ allows the C-terminal portion of the protein to be released to the cell wall towards the end of the lytic cycle by the pore-forming holins to degrade the PG layer in conjunction with the Tuc2009 endolysin, resulting in a more efficient cell lysis. It is also possible that multiple molecules of both the complete and C-terminally truncated Tal$_{2009}$ protein become incorporated into the phage upon assembly of the mature virion. Previous work (Seegers et al., unpublished) has shown that C-terminally truncated Tal$_{2009}$ does indeed become incorporated into the phage. The purpose of this inclusion is as yet unknown, although it is probably a more likely reason for the autoproteolysis than the production of an auxiliary player in cell lysis. It may be that the stoichiometry or spatial arrangement of the tail tip proteins requires that some versions of Tal$_{2009}$ incorporated into the phage do not have an additional 30 kDa on the C terminus of the protein.

Direction of Tal$_{2009}$ to the target bond is probably caused by the spatial arrangement of the catalytic domain at the PG upon cell binding by the phage. In support of this theory, it was demonstrated that Tal$_{2009}$ does not bind the cell wall. This omission of a binding domain probably decreases the lytic

activity of Tal_{2009}, which may be a deliberate property given the requirement for minimal disruption to the viability of the host cell upon infection initiation. The location of lytic activity in Tal_{2009} to one end of the protein promotes the idea of exposure of this zone at the end of the phage tail. The theory of phage evolution by exchange of functional modules has already been well articulated in terms of the whole genome (7) and the tail fibers (48) and down to the level of N- and C-terminus functionality of lysins (16). It has been suggested that homologous recombination can occur within DNA sequences specifying distinct protein domains (39). Gly-rich collagen-like repeats of phage structural proteins have been proposed as sites for gene reshuffling (17). Our results indicate that tail-associated lysins can follow the same mosaic pattern of functional modules of proteins. Indeed, preliminary work in this laboratory indicates that this is also true for tail-associated lysins of other phages which infect gram-positive bacteria.

To determine whether Tal_{2009} was necessary for phage infection we performed Ab neutralization experiments, an approach which has been used previously for lactococcal phages (11, 23). Initially tests were performed using an MOI of 0.02 for c2 and Tuc20009 with their respective hosts. Upon observing a >2 log difference in UC509.9 cell numbers in the presence or absence of Tal_{2009}-specific Abs it was decided to increase the stringency of the test and drive cell lysis by using a MOI of 20 for Tuc2009 with UC509.9. Once again we observed a >2 log reduction of UC509.9 cell numbers when Tal_{2009}-specific Abs were not present (Fig. 5A). No effect was observed for lysis of MG1614 by the bacteriophage c2 (Fig. 5B). However, we cannot exclude the possibility that binding of the Abs to Tal_{2009} elicits some sort of stearic hindrance to other phage-encoded proteins.

Our findings show that the tail lysin is located at the tip of the phage tail, which indicates that the Tuc2009 DNA infection process involves the action of a lysin, possibly to allow safe passage of a DNA injection device to the cell membrane. In an effort to more fully elucidate this mechanism and to explain the apparent disparity between the substrate spectrum and bond recognition seen in autoproteolysis, further studies of Tal_{2009} are in progress.

ACKNOWLEDGMENTS

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