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Molecular Characterization of Lactococcal Bacteriophage Tuc2009 and Identification and Analysis of Genes Encoding Lysin, a Putative Holin, and Two Structural Proteins

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Bacteriophage Tuc2009 is a temperate bacteriophage with a small isometric head and is isolated from *Lactococcus lactis* subsp. *cremoris* UC509. The phage genome is packaged by a headful mechanism, giving rise to circularly permuted molecules with terminal redundancy. The unit genome size is approximately 39 kb. A map of the phage genome on which several determinants could be localized was constructed: *pac*, the site of initiation of DNA packaging; *lys* (1,287 bp), specifying the phage lysin; *S* (267 bp), specifying a putative holin; and *mp1* (522 bp) and *mp2* (498 bp), each specifying one of the phage's structural proteins. *lys*, *S*, *mp1*, and *mp2* were further characterized. *lys* and *S* are partially overlapping and appear to be part of one operon. The lysin shows homology to the lysins of the *Streptococcus pneumoniae* phages Cp-9, Cp-1, and Cp-7. The putative holin, which is thought to be involved in the release of lysin from the cytoplasm, contains two strongly hydrophobic presumptive transmembrane domains and a highly charged C-terminal domain.

The genus *Lactococcus* belongs to the relatively small but economically significant group of lactic acid bacteria that play a key role in the production of fermented milk products (18). They not only serve to preserve the products but also contribute to the development of flavor and texture. In the dairy industry in particular, in which large-scale fermentations have now become common, the control of bacteriophages which could disrupt the fermentation process is of prime importance. A thorough knowledge of the bacteriophage and bacteriophage-host relationship at the molecular level will open the way to the development of new strategies for bacteriophage defense. Moreover, it will yield invaluable information on the regulation of gene expression in lactococci, which may find application in future fermentations, where it will serve to improve the overall control of the process as well as the expression of particular homologous or heterologous genes.

The mechanisms of cell lysis by bacteriophages have been particularly well investigated in gram-negative bacteria (for a review, see reference 36). Although some bacteriophages have evolved alternative systems, a generalized model involves the action of two principal genes. According to this model, a holin (36) mediates the transport of a lysin across the cytoplasmic membrane, which subsequently degrades the bacterial cell wall. Recently, Steiner et al. (25) demonstrated the presence of analogous functions in the *Bacillus subtilis* bacteriophage φ29.

Lysin genes of bacteriophages of lactic acid bacteria that have been cloned to date include those of the *Lactococcus lactis* bacteriophages φvML3 (23), c2 (35), and φUS3 (21), and the *Lactobacillus bulgaricus* bacteriophage mv1 (3). Approximately 150 and 35 bp upstream of the lysin sequences of φUS3 and mv1, respectively, open reading frames (ORFs) which may encode holin-type proteins have been identified (21, 36). The φvML3 lysin could be expressed in *L. lactis* without any effect on exponentially growing cells (24), also suggesting the need for a phage-encoded lysin export function to effect lysis of the host. Unlike the situation with φUS3 and mv1, the holin and lysin genes of bacteriophage Tuc2009 described in this report appear to be configured in a manner similar to that in the lambdoid phages, where they overlap each other by one or more base pairs (36).

Two genes that encode structural proteins were localized on the phage Tuc2009 genome and characterized at the DNA sequence level. For the proteins specified by these genes, no homologous counterparts could be found in data base searches.

MATERIALS AND METHODS

Bacteriophage, bacterial strains, and plasmids. The bacteriophage, bacterial strains, and plasmids used in this study are listed in Table 1. Plasmid pSK+203 contains a 13.7-kb Tuc2009 *Pvu*II fragment cloned into the *Eco*RV site of pBluescriptIISK+ (Stratagene, La Jolla, Calif.). DNA sequence analysis revealed that the lysin-coding sequence on this Tuc2009 fragment is flanked by a *Pst*I site at the 5′ end (within the putative holin-coding sequence [see Fig. 3]) and by an *Eco*RV site at the 3′ end (0.7 kb downstream of the lysin sequence). By using *Escherichia coli* XL1Blue as the host for transformation, the *Pst*I-*Eco*RV fragment containing the lysin sequence was cloned into pBluescriptIISK− (Stratagene) in order to provide the fragment with suitable restriction sites for the next cloning step. The lysin sequence (including the ribosomal binding site) was subsequently excised from the resulting plasmid pKS-lys with *Bam*HI and *Sal*I and cloned downstream of the T7 *lac* promoter in pET24 (Novagen,

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TABLE 1. Phage, bacterial strains, and plasmids

Phage, bacterial strain, or plasmid	Relevant feature(s)	Source and/or reference(s)
Phage Tuc2009	Isolated after induction from *L. lactis* UC509	6, 9
Bacteria		
L. lactis subsp. *cremoris*		
UC509	Lysogenic host of Tuc2009	9
UC526	Indicator strain for Tuc2009	9
E. coli		
XL1Blue	*recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1* (F′ *proAB lacI*^{q} *lacZ*ΔM15 Tn*10*)	Stratagene
BL21(DE3)	F^- *ompT* r_B^- m_B^- (DE3), λDE3 lysogen, carrying the T7 RNA polymerase gene under *lacUV5* control	Novagen (26)
Plasmids		
pBluescriptIISK+	Amp^r, *lacZ* α complementation	Stratagene
pSK+203	Amp^r, pBluescriptIISK+, carrying a 13.7-kb Tuc2009 *Pvu*II fragment	This work
pBluescriptIIKS−	Amp^r, *lacZ* α complementation	Stratagene
pKS-lys	Amp^r, pBluescriptIIKS−, carrying the Tuc2009 lysin sequence on a 2.1-kb *Pst*I-*Eco*RV fragment	This work
pET24	Km^r, containing a T7 *lac* promoter (T7 promoter and *lac* operator)	Novagen
pET24lys	Km^r, pET24 carrying the Tuc2009 lysin sequence downstream of the T7 *lac* promoter	This work

Madison, Wis.) to generate pET24lys. *E. coli* BL21(DE3) was used as the host for transformation in this experiment.

Media. *L. lactis* was grown in LSB (a modified version of M17 [27] which contains per liter, 10 g of lactose, 10 g of meat extract, 5 g of yeast extract, 5 g of tryptone, 5 g of tryptose, and 7.2 g of sodium-β-glycerophosphate) or on LSB solidified with 1.5% agar. *E. coli* was grown in TY broth (22) or on TY solidified with 1.5% agar, supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 μg/ml), and isopropyl-β-D-thiogalactopyranoside (IPTG) (0.5 mM) where appropriate.

Induction and propagation of bacteriophage Tuc2009. Bacteriophage Tuc2009 was induced from its lysogenic host *L. lactis* subsp. *cremoris* UC509. Strain UC509 was grown at 30°C to an optical density of 0.25 (at 600 nm), after which mitomycin was added to a final concentration of 2.5 μg/ml. Incubation was continued for an additional 4 to 6 h by which time lysis had occurred. Subsequently, the phage was propagated on its permissive host *L. lactis* subsp. *cremoris* UC526, which allowed multiplication of the phage to a higher titer. In this case, $CaCl_2$ was added to a final concentration of 10 mM. Phages were concentrated and purified by CsCl gradient centrifugation as described by Fitzgerald et al. (11).

Electron microscopy. Phages were negatively stained with 2% uranyl acetate and examined in a JEOL 1200 EX transmission electron microscope (JEOL, London, United Kingdom) at an accelerating voltage of 80 kV.

DNA manipulations. Bacteriophage DNA was isolated as described by Fitzgerald et al. (11). Plasmid DNA was isolated by the method of Birnboim and Doly (2). Restriction enzymes and T4 DNA ligase were purchased from Boehringer GmbH (Mannheim, Germany) and used according to the instructions of the supplier. The phage genome was cloned into the *E. coli* vector pBluescriptIISK+ (Stratagene) by shotgun cloning of *Eco*RV- or *Pvu*II-generated fragments. Oligonucleotides were synthesized with an Applied Biosystems 391 DNA synthesizer (Applied Biosystems Inc., Foster City, Calif.).

DNA sequence analysis. DNA sequence analysis was performed with an Applied Biosystems 373A automated DNA sequencer. Bacteriophage Tuc2009 sequences were obtained by sequencing of plasmids containing cloned Tuc2009 fragments and by direct sequencing of the phage DNA. The combination of results obtained following this procedure and restriction endonuclease mapping of cloned fragments allowed the construction of a map of the phage genome. Data base searches were performed with the programs FASTA (20) and BLAST (1).

SDS-PAGE and determination of N-terminal amino acid sequences of structural phage proteins. Concentrated and purified phage particles were boiled in sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16) and applied to SDS–12% polyacrylamide gels (mini-protean II; Bio-Rad Laboratories, Richmond, Calif.). After electrophoresis, the proteins were either visualized by staining with Coomassie brilliant blue or transferred to ProBlott membranes (Applied Biosystems). The blots were stained with Coomassie brilliant blue, protein bands were excised, and their N-terminal amino acid sequences were determined with an Applied Biosystems 477A protein sequencer.

Expression of the Tuc2009 lysin in *E. coli*. *E. coli* BL21 (DE3) containing either pET24lys or pET24 was grown to an optical density at 580 nm of 0.4, after which IPTG was added to a final concentration of 0.4 mM to induce the production of T7 RNA polymerase and, consequently, the lysin. Cultures were incubated for 1 h at 37°C, after which rifampin was added to a final concentration of 200 μg/ml. After incubation for an additional 1 h, the cells were collected by centrifugation and washed in 50 mM Tris-HCl (pH 7.0). Cell pellets were stored at −20°C at this stage. Before SDS-PAGE, the cells were resuspended in 1/200 volume of H_2O, and an equal volume of 2× sample buffer (16) was added. The samples were heated to 100°C for 5 min and applied to a 10% polyacrylamide gel. After electrophoresis, the proteins were visualized by staining with Coomassie brilliant blue.

Assay of lysin activity. In order to confirm lysin activity, *E. coli* BL21(DE3) containing either pET24lys or pET24 was grown as described above. IPTG was added to a final concentration of 3 mM, and after 3 h, the cells were collected by centrifugation. No rifampin was added. The cells were washed in 66 mM potassium-phosphate buffer (pH 6.2), resuspended in 1/60 volume of the same buffer, and disrupted with a Shake it, Baby cell disrupter (Biospec Products, Bartlesville, Okla.), as described by Van de Guchte et al. (33). Cell debris was

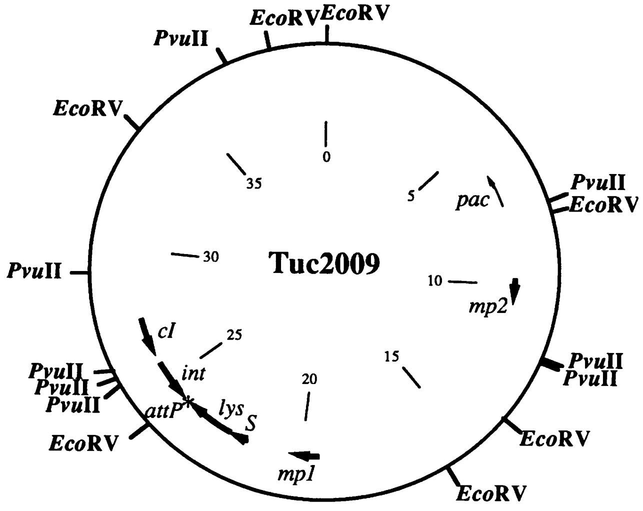

FIG. 1. Genomic map. Restriction map of the Tuc2009 genome, on which the locations of a number of determinants are indicated. The genome size is approximately 39 kb. *pac* is the site of initiation of DNA packaging, and the thin arrow indicates the predicted direction of packaging. *attP* is the site of recombination with the host chromosome. *int* encodes the phage integrase. *lys* encodes the phage lysin. *S* encodes a putative holin. *mp1* and *mp2* encode major proteins of the phage. *c*I encodes the putative phage repressor. *int* encodes the phage integrase. *attP* is the site of recombination with the host chromosome. Size coordinates given in the genomic map are in kilobases.

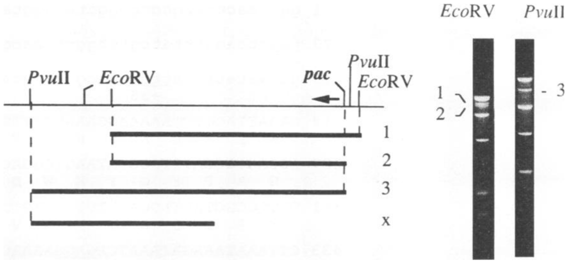

FIG. 2. Localization of the *pac* site and direction of packaging. Tuc2009 DNA digested with *Eco*RV and *Pvu*II. 1, 2, and 3 indicate submolar fragments, and the bars indicate their relative positions on the Tuc2009 map. The two *Pvu*II sites indicated on the map are approximately 10.5 kb apart. *pac* is the site of initiation of DNA packaging. The arrow indicates the predicted direction of packaging. For explanation of fragment x, see the text.

subsequently removed by centrifugation in an Eppendorf centrifuge, and the supernatant was assayed for lysin activity. To this end, a culture of *L. lactis* UC526 grown overnight was diluted 1:10 in fresh LSB and incubated for 5 h at 30°C. The cells were autoclaved and collected by centrifugation, washed in 66 mM potassium-phosphate buffer (pH 6.2), and resuspended in the same buffer to an optical density at 450 nm of 0.6. The *E. coli* cell extracts were added to the UC526 cell suspension, and the optical density at 450 nm was monitored over time.

RESULTS

Morphology and propagation. Tuc2009 is a phage with a small isometric head (diameter, 52 nm), a noncontractile tail (152 nm long), and a baseplate (width, 16 nm). The phage was first isolated after induction from its lysogenic host *L. lactis* subsp. *cremoris* UC509, one of the bacterial strains constituting a commercially available mixed strain starter culture in use in an Irish Cheddar cheese factory in 1982 (9). The phage was subsequently shown to multiply in a lytic fashion on several other industrial *L. lactis* strains (9), including *L. lactis* subsp. *cremoris* UC526 (9). This host was used to propagate the phage prior to isolating DNA for use in cloning experiments.

Restriction map of the Tuc2009 genome, headful mechanism of packaging, and location of the *pac* site. The Tuc2009 phage genome was cloned in *E. coli* with the pBluescriptII vector system, and its DNA sequence is being determined. Sequencing and restriction endonuclease mapping of the cloned fragments, together with direct sequencing of the phage DNA, allowed the construction of a map of the phage genome (Fig. 1; in order to provide a more complete picture of the phage genome, the following genes in addition to those described in this report have been included in the map: *c*I, which specifies the putative phage repressor; *int*, which specifies the phage integrase; and *attP*, which is the site of recombination with the host chromosome [29, 30]). The unit genome size is approximately 39 kb.

When Tuc2009 DNA was digested with different restriction enzymes, one or two fragments appeared to be present in submolar amounts. The same result was obtained whether or not the restricted DNA was heated to 65°C before electrophoresis, and the restriction pattern did not change when the DNA was ligated prior to digestion (6). These results indicated that Tuc2009 DNA had no *cos* site but, instead, was packaged by a headful mechanism. In an *Eco*RV digest of Tuc2009 DNA (Fig. 2), two submolar fragments of approximately 8.5 kb were recognized, of which one could be cloned and proved to be delineated by an *Eco*RV site at either end (Fig. 2, fragment 1). Only one *Eco*RV fragment of this size is present in the Tuc2009 genome, and its submolarity can be explained by assuming that the *pac* site is situated on this fragment. The second, slightly smaller, fragment (Fig. 2, fragment 2) is assumed to be delineated by the *pac* site at one end and by an *Eco*RV site at the other end. An overlapping submolar fragment was found to be present in *Pvu*II digests of Tuc2009 DNA (Fig. 2, fragment 3). Additional support for a headful mechanism of packaging came from the cloning of a DNA fragment which mapped near the proposed *pac* site (fragment x in Fig. 2). Although obtained from *Pvu*II-digested Tuc2009 DNA, this fragment was not visible in a *Pvu*II digest. DNA sequence analysis revealed that it had been generated by *Pvu*II cleavage at only one end, while the other end bore no resemblance to a *Pvu*II recognition site. We speculate that the latter end represents a site where the packaging of one phage head had been completed and the packaging of another phage head started.

Identification of the Tuc2009 *lys* gene. Examination of the Tuc2009 DNA sequence identified an ORF designated *lys* which encodes the phage lysin (Fig. 3). The deduced amino acid sequence, which contains 428 residues, shows homology to the lysins of the *Streptococcus pneumoniae* phages Cp-9 (in 331 amino acids, 68% similarity and 26% identity), Cp-1, and Cp-7 (12) and to a lesser extent to the lysin of the *Lactobacillus bulgaricus* phage mv1 (3) (in 159 amino acids, 68% similarity and 26% identity). As observed with other lysins (36), including that of phage mv1, a hydrophobic domain could be recognized in the N-terminal region of the protein (Fig. 4).

A consensus Shine-Dalgarno (SD) sequence (19, 32) precedes the lysin-coding sequence. A strong transcriptional terminator ($\Delta G = -25.0$ kcal/mol [28]) is present downstream of and overlapping the 3′ end of the coding sequence (Fig. 3),

```
   1 ggctcacttaagcctgcggttataggtatagattgcgctcaagcaccacccagttggttcagtagtcacaaa
                                                          -35
  73 taactcaaatttatcgtctggctacacgactggcacatggcagaacatcggttcagcagtaattggttcaac
     -10                                   -35                 -10
 145 gacaatatattattggcaacgcactgcataaaaatataaaaaataggagagtaaaATGAATCAAATCAATTG
                  -35                   -10       SD         M  N  Q  I  N  W
 217 GAAATTACGTTTAAAAAGCAAAGCTTTTTGGTTAGCTTTACTACCTGCTCTATTCTTGCTAATACAAGCTAT
       K  L  R  L  K  S  K  A  F  W  L  A  L  L  P  A  L  F  L  L  I  Q  A  I
 289 AGGAGCGCCATTTGGCTATAAGTGGGACTTTGTTATTTTAAATCAACAACTTGCTGCAGTGGTTAATGCTGC
       G  A  P  F  G  Y  K  W  D  F  V  I  L  N  Q  Q  L  A  A  V  V  N  A  A
 361 TTTTGCGCTATTAGCAATTGTTGGAGTTGTTGCTGACCCAACGACCAGTGGTCTAGGAGATAGTGATAGAGT
       F  A  L  L  A  I  V  G  V  V  A  D  P  T  T  S  G  L  G  D  S  D  R  V
 433 CTTAAATAAAGATAAATCAGAGGAAAACAAATGAAAAGATTAATCAAAAAATCTGCCATTGGAATGTTCGCT
       L  N  K  D  K  S  E  E  N  K  -
                         SD         M  K  R  L  I  K  K  S  A  I  G  M  F  A
 505 TTCTTTGTTGTTGCAGCAAGTGGACCTGTATTTGCGGCATCCGGTGACCAAGGTGTGGACTGGTCAAAATAT
      F  F  V  V  A  A  S  G  P  V  F  A  A  S  G  D  Q  G  V  D  W  S  K  Y
 577 AACGGAACTTACGGTAATTTTGGTTATGCTCATGATAAATTTGCTTTTAGCCAAATCGGAGGAACTTACGGT
      N  G  T  Y  G  N  F  G  Y  A  H  D  K  F  A  F  S  Q  I  G  G  T  Y  G
 649 GGAACCTTTGTAGACCAAGCCACCTATAAAACGCAAGTAGCTTCAGCAATTGCTCAAGGTAAACGAGCGCAC
      G  T  F  V  D  Q  A  T  Y  K  T  Q  V  A  S  A  I  A  Q  G  K  R  A  H
 721 ACTTACATTTGGTATCAAGTCGGAGGTTCGCAAGAAGTAGCAAAAGCAGCACTTGATCGTTACTTACCAAAA
      T  Y  I  W  Y  Q  V  G  G  S  Q  E  V  A  K  A  A  L  D  R  Y  L  P  K
 793 ATTCAAACGCCTAAAAACTCTATTGTTGCTTTGGACTATGAAAGTGGAGCAAGTGGAGATAAACAAGCAAAT
      I  Q  T  P  K  N  S  I  V  A  L  D  Y  E  S  G  A  S  G  D  K  Q  A  N
 865 ACTGATGCGATTCTTTACGGAATGCGTCGAGTAAAAGCGGCTGGATATACTCCAATGTATTATTCTTACAAG
      T  D  A  I  L  Y  G  M  R  R  V  K  A  A  G  Y  T  P  M  Y  Y  S  Y  K
 937 CCTTACACTTTGGCCAATGTTAATTATAAGCAAATCATCAAAGAATTCCCTAACTCATTATGGATTGCGGCA
      P  Y  T  L  A  N  V  N  Y  K  Q  I  I  K  E  F  P  N  S  L  W  I  A  A
1009 TATCCCAATTATGAAGTAACACCAGTTCCAAACTATAGCTTCTTCCCAAGTATGGACGGAATATCATTATTT
      Y  P  N  Y  E  V  T  P  V  P  N  Y  S  F  F  P  S  M  D  G  I  S  L  F
1081 CAGTTCACATCCACTTATATCGCTGGTGGACTGGATGGTAATGTTGATTTAACAGGAATCACAGATAATGGA
      Q  F  T  S  T  Y  I  A  G  G  L  D  G  N  V  D  L  T  G  I  T  D  N  G
1153 TACAGAAAACAGAAAGGCCAAGAAGTTAAACCCAATACTGCTACACCGGCCATTGAAAATGGTAAAGAAGCC
      Y  R  K  Q  K  G  Q  E  V  K  P  N  T  A  T  P  A  I  E  N  G  K  E  A
1225 AATGAAGTTAAAGGAAACGATGTAGAAGCTGGAATGACGGTTAAAGTAAACTTTGGCGCTAAGAATTATGCC
      N  E  V  K  G  N  D  V  E  A  G  M  T  V  K  V  N  F  G  A  K  N  Y  A
1297 ACAGGAGAAACAATTCCTCAATGGGTAAAAGGTCAACCACATAAAATCATCCAGAAGAATGGAGATACTGTC
      T  G  E  T  I  P  Q  W  V  K  G  Q  P  H  K  I  I  Q  K  N  G  D  T  V
1369 TTGCTTGATGGTATTATGAGCTGGTTATCCGTTCATGATGTGGAAACTATTGATTCTACAAGCCAGCCAACG
      L  L  D  G  I  M  S  W  L  S  V  H  D  V  E  T  I  D  S  T  S  Q  P  T
1441 ACACCCGCAAAAAGTTATGTTGTAAAACAAGGTGATACACTTAGTGGCATTGCTTCAAACTGGGGTACTAAC
      T  P  A  K  S  Y  V  V  K  Q  G  D  T  L  S  G  I  A  S  N  W  G  T  N
1513 TGGCAAGAATTAGCACGTCAGAACAGTTTATCTAACCCGAACATGATTTATGCAGGTCAGGTTATTAGCTTC
      W  Q  E  L  A  R  Q  N  S  L  S  N  P  N  M  I  Y  A  G  Q  V  I  S  F
1585 ACAGGCGGTCAATCTGGGGCTACAGCACGAGCTTACACTGTACAATCTGGCGATAATCTTTCATCAATTGCG
      T  G  G  Q  S  G  A  T  A  R  A  Y  T  V  Q  S  G  D  N  L  S  S  I  A
1657 ATCCTTTTAGGAACAACAGTTCAAAGCTTAGTTTCAATGAATGGTATTTCAAACCCTAATTTGATTTACGCT
      I  L  L  G  T  T  V  Q  S  L  V  S  M  N  G  I  S  N  P  N  L  I  Y  A
1729 GGTCAAACACTAAATTATTAAaattaaccccgcttcggcgggtgtttttttaaatataatttattcaaataa
      G  Q  T  L  N  Y  -
```

FIG. 3. Nucleotide sequence of the phage Tuc2009 *S-lys* region. *S* runs from nucleotides 200 to 466. *lys* runs from nucleotides 463 to 1749. The inferred amino acid sequences are given below the DNA sequence. Putative −35 and −10 promoter sequences, as well as SD sequences and start codons (ATG) are underlined. The inverted repeat constituting a putative transcription terminator is doubly underlined. The *Pst*I recognition sequence (CTGCAG) used for cloning of the lysin sequence in pET24 is printed in boldface type. GS data base accession number L31364.

which is adjacent to the bacteriophage attachment site, *attB* (29).

Expression of the *lys* gene in *E. coli*. In order to confirm its function, the lysin-coding sequence (including ribosomal binding site) was cloned under the control of a T7 *lac* promoter in the plasmid pET24lys and subsequently expressed after induction of the T7 RNA polymerase in *E. coli* BL21(DE3) containing this plasmid. SDS-PAGE revealed the presence of a 49-kDa protein in BL21(DE3) containing pET24lys, which is not present in the strain containing pET24 (Fig. 5). This result is in accordance with the predicted molecular weight of 46,300 for the Tuc2009 lysin. The protein was found to be active against *L. lactis* UC526, as judged by the decrease in the optical density of an UC526 cell suspension after the addition of the *E. coli* BL21(DE3)(pET24lys) extract. No decrease was observed following the addition of an extract of BL21(DE3) containing pET24 (results not shown).

Identification of *S*, a putative holin-coding sequence. Analysis of the region upstream of the lysin-coding sequence, showed the presence of another ORF, designated *S*, which we speculate may encode a holin involved in the release of lysin by permeabilization of the cytoplasmic membrane. As in other holins, two strongly hydrophobic potential transmembrane domains as well as a highly charged C-terminal domain can be recognized in the deduced amino acid sequence (Fig. 6). The holin-coding sequence (267 bp, capable of encoding a protein of 88 amino acids), is preceded by a consensus SD sequence, which in turn is closely preceded by near-consensus −35 and −10 promoter sequences (Fig. 3). Other putative promoter sequences are situated further upstream.

The presumptive Tuc2009 holin shows a high degree of homology (in 84 amino acids, 69% similarity and 50% identity) to the protein potentially encoded by an ORF preceding the lysin-coding sequence in the *S. pneumoniae* phage EJ-1 (10) (ORF2) (Fig. 6). We speculate that this highly homologous ORF in EJ-1 also encodes a holin. In contrast, the lysins of

```
1                                MVKKNDLFVDVASHQGYDISGILEEAGTTNTIIKVSESTSYL
                                     :    : :  :     :    :    ¦        :
2   MKRLIKKSAIGMFAFFVVAASGPVFAASGDQGVDWSKYNGTYGNFGYAHDKFAFSQIGGTYGGTFVDQATY
              :     :   :   :      :       :        :     :  :   ¦   ¦ ¦
3        MDIDTSRLRTDLPQVGVQPYRQVHAHSTGNRNSTAQNEADYHWRKDPELGFFSHVVGNGRVMQVGP

1   NPCLSAQVSQSNPIGFYHFACFGGNEEEAEAEARYFLDNVPTQVKYLV-LDYEDHASASVQRNTTACLRFM
       ::: ::¦:     ¦     ¦¦ :¦ ¦ ¦    :¦  : ¦    :¦ ¦¦¦¦  ¦¦   ¦ ¦¦ ¦ ¦  ¦
2   KTQVASAIAQGKRAHTYIWYQVGGSQEVAKAALDRYLPKIQTPKNSIVALDYESGASGDKQANTDAILYGM
      : :  :: ¦  ¦ ¦¦   ::  ¦       :: ¦   ¦:  :¦     ¦       ¦  ¦   :  ¦
3   VNNGSWDVGGGWNAETYAAVELIESHSTEEEFMEDYRLYIELLRNLADEADLPKTLDTDDLAGIKTHEYCT

1   QMIAEAGYTPIY-YSY-KPFTLDNVDYQQILAQFPNSLWIAGYGLNDGT--ANFEYFPSMDGIRWWQYSSN
      :  ¦¦¦¦¦:¦ ¦¦¦ ¦¦:¦¦ ¦¦:¦ ¦¦: :¦¦¦¦¦¦¦¦:¦   : ¦  :¦: :¦¦¦¦¦¦¦   ¦::¦
2   RRVKAAGYTPMY-YSY-KPYTLANVNYKQIIKEFPNSLWIAAYPNYEVTPVPNYSFFPSMDGISLFQFTST
              :  ¦ ¦     ::  ::¦¦ ¦ :
3   NNQPNNNSDHVDPYPYLAKWGVSREQFKQDIEN

1   PFDKNIVLLDDEKEDNINNENTLKSLTTVANEVIQGLWGNGQERYDSLANRGYDPQAVQDKVNEILNAREI
         :    ¦            ¦:    :¦    ::    :       :         :       : ¦
2   YIAGGLDGNVDLTGITDNGYRKQKGQEVKPNTATPAIENGKEANEVKGNDVEAGMTVKVNFGAKNYATGET

1   ADLTTVANEVIQGLWGNGQERYDSLANRGYDPQAVQDKVNEILNAREIADLTTVANEVIQGLWGNGQERYD
          ::              ¦::       :           :   : :   :: :       ¦ :  :
2   IPQWVKGQPHKIIQKNGDTVLLDGIMSWLSVHDVETIDSTSQPTTPAKSYVVKQGDTLSGIASNWGTNWQE

1   SLANRGYDPQAVQDKVNELLS
       : :     :    : :
2   LARQNSLSNPNMIYAGQVISFTGGQSGATARAYTVQSGDNLSSIAILLGTTVQSLVSMNGISNPNLIYAGQ

2   TLNY
```

FIG. 4. Alignment of lysins of three bacteriophages. Sequences: 1, *S. pneumoniae* phage Cp-7; 2, *L. lactis* phage Tuc2009; 3, *S. pneumoniae* phage EJ-1 (N-terminal part only). Pairwise alignments to the Tuc2009 sequence are shown. Identical amino acids (¦) and conservative replacements (:) are indicated. No satisfactory alignment for the C-terminal part of the EJ-1 lysin was found. The strongly hydrophobic domain is underlined.

both phages could be aligned only to some (low) extent in their N termini (Fig. 4). The sequence of lysin of Tuc2009 aligned very well with those of Cp-9, Cp-1, and Cp-7 (Fig. 4) in their nearly identical (12) N-terminal domains, and in the case of Cp-9 also to a considerable extent in its C-terminal domain (results not shown). Inspection of the Cp-7 DNA sequence presented by García et al. (12) showed the presence of (the 3′ end of) an ORF directly upstream of and overlapping the lysin-coding sequence. The inferred amino acid sequence showed only a very low degree of homology to the Tuc2009 holin sequence, however (Fig. 6). Furthermore, there was no clear indication of structural similarity between the Cp-7 protein and proteins of the holin family (data not shown).

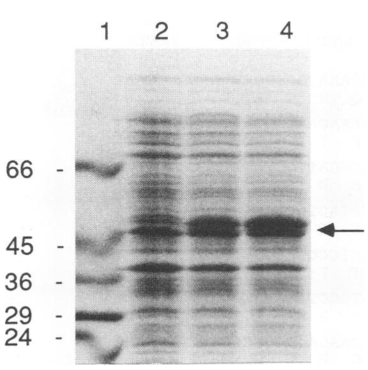

FIG. 5. Expression of the Tuc2009 *lys* gene in *E. coli*. Samples of *E. coli* BL21 (DE3) were subjected to SDS-PAGE (10% polyacrylamide). Lanes: 1, molecular size standards (Sigma High Molecular Weight Standard Mixture; Sigma, St. Louis, Mo.) (molecular sizes are given in kilodaltons); 2, BL21(DE3)(pET24); 3 and 4, BL21(DE3)(pET24lys). The arrow indicates the position of the Tuc2009 lysin.

Structural phage proteins. The separation of Tuc2009 phage proteins by SDS-PAGE revealed the presence of three major proteins and a few minor proteins, of which the most abundant (MP4) had an estimated molecular weight of 32,000 (Fig. 7). The N-terminal amino acid sequences of MP4 and three other proteins (MP1, MP2, and MP3, with molecular weights of 19,000, 22,000, and 29,000, respectively) were determined and are as follows:

MP4, SerLysGlnLysThrThrLeuThrAspLeuValAsnTrpArgVal;
MP3, MetAspLeuLeuIleThrIleThrGlnAsnGlu;
MP2, AlaGluLeuThrAlaLysGlnGlyLysAspIleIleLeuLeuTyrArgLeuLeuSerLysAla;
MP1, AlaGluLeuThrLysIleThrArgGlyMetGlnAsnGlyAla

```
1             SWTGLKGVIKHTLTFIFYYFVAVFLTYIHAMAVGQILLVIIN
                 :    :  :¦  :     ¦ ¦      : : ¦ ¦  ::¦
2   MNQINWKLRLKSKAFWLALLPALFLLIQAIGAPFGYKWDFVILNQQLAAVVN
       ¦¦¦¦¦¦¦¦ ¦ ¦¦¦: :¦¦  ¦::¦¦ :¦ ¦¦¦  ¦   : : ¦  :¦¦
3     MINWKLRLKNKFFWLTAIPAFLLVLQAGAAVFGYHLDLGDIGNKLILLVN

1   LYYALSIMENLAVMGVFIPKFMTARVQEELQKYTAQLDAGKDLLEEFKGEKK
      :¦¦  :  :             ¦¦ :
2   AAFALLAIVGVVADPTTSGLGDSDRVLNKDKSEENK
    ¦ ¦  ¦: :¦:¦ ¦¦¦¦¦¦::¦¦ ¦ ¦:  ¦: ¦:
3   AVFVFLTAIGLVNDPTTSGIADSTRALEYKKPSEE
```

FIG. 6. Amino acid sequences derived from ORFs preceding lysin-coding sequences. Sequences: 1, *S. pneumoniae* phage Cp-7 (N-terminal part of sequence not known); 2, *L. lactis* phage Tuc2009; 3, *S. pneumoniae* phage EJ-1. Pairwise alignments to the Tuc2009 sequence are shown. Identical amino acids (¦) and conservative replacements (:) are indicated. Strongly hydrophobic domains (putative transmembrane domains) are underlined, and the highly charged C-terminal domain is italicized.

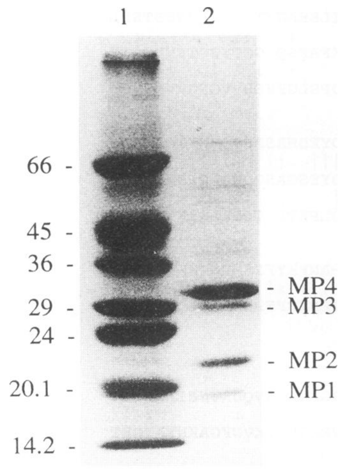

FIG. 7. Bacteriophage Tuc2009 proteins. Separation of bacteriophage Tuc2009 proteins by SDS-PAGE. Lane 1, molecular size standards (Dalton Mark VII-L; Sigma) (molecular sizes are given in kilodaltons); lane 2, Tuc2009 proteins. Proteins of which the N-terminal amino acid sequences were determined (MP1 to MP4) are indicated.

The first four amino acids of MP1 were found to be the same as those of MP2. Only MP3 appeared to have a methionine as the first residue, whereas the N-terminal formylmethionine had been removed from the other proteins. DNA and protein sequencing data were combined to identify the ORFs encoding the 19- and 22-kDa proteins, *mp1* and *mp2*, respectively. These two ORFs are separated by a DNA sequence of approximately 9 kb (Fig. 1).

mp1 (Fig. 8) potentially encodes a neutral protein (isoelectric point [pI], 7.3) of 172 residues (after cleavage of the N-terminal formylmethionine) with a predicted molecular weight of 18,800, which agrees very well with the molecular weight of 19,000 estimated by SDS-PAGE. Putative promoter and SD sequences are present upstream of the coding region. No transcription terminator was detected directly downstream of this gene. Instead, other ORFs could be found (data not shown), indicating that *mp1* is part of an operon.

mp2 (Fig. 9) potentially encodes an acidic protein (pI, 4.6), which (after cleavage of the N-terminal formylmethionine) contains 164 residues and has a predicted molecular weight of 18,100. This result is at variance with the data from SDS-PAGE, where MP2 appeared to be bigger than MP1, with an estimated molecular weight of 22,000. The *mp2* sequence is directly preceded by an ORF, *x*, potentially encoding a 14.8-kDa neutral protein (pI, 7.3) (Fig. 9). The two ORFs are in close proximity, in a configuration in which the stop codon of ORF *x* overlaps the SD sequence of *mp2*. Putative SD sequences were identified upstream of ORF *x* and *mp2*, while putative promoter sequences could also be identified upstream of ORF *x*. Only a very weak potential transcription terminator ($\Delta G = -8.4$ kcal/mol [28]) could be found downstream of *mp2* (Fig. 9). Data base searches with the programs FASTA (20) and BLAST (1) did not reveal any sequences with significant homology to protein MP1 or MP2 or the protein potentially encoded by ORF *x*. However, MP2 and the ORF *x* protein did show some resemblance to viral coat proteins (results not shown).

DISCUSSION

The results obtained by electron microscopy showed that the temperate *L. lactis* bacteriophage Tuc2009 possesses a small isometric head, a noncontractile tail, and a baseplate. Tuc2009 therefore belongs to the family *Siphoviridae* and to morphotype B according to the classification of Bradley (4). According to the taxonomy proposed by Jarvis et al. (13), Tuc2009 is possibly a member of the P335 species, of which phage P335 (5) is the type phage. As pointed out by these researchers, DNA homology studies are required to confirm this classification.

Most lactococcal bacteriophages which have been characterized at a molecular level appear to have a *cos* site (15), to ensure that exactly one entire phage genome is packaged into each phage head. However, the temperate *L. lactis* subsp. *cremoris* phage described here, like the *L. lactis* subsp. *cremoris* phage BK5-T (17) and the *Lactobacillus delbrueckii* phages LL-H and mv4 (34), belongs to a class of phages in which more than a unit-size genome is packaged into each phage head by a so-called headful mechanism. According to this model (7), a concatemeric molecule undergoes site-specific cleavage at the *pac* site and packaging occurs from this point to a (undefined) point beyond the next *pac* site, where the packaging of the next

```
  1 CAAATAAATGGCAATGTTGAAAGCGACAAGTCAGAAAGTGTTGCTTTTAAAACAACACTACCCAAATAATAG
                     -35                           -10
 73 AAATAGGAGAATAAAATGGCTGAATTAACTAAAATTACTCGAGGTATGCAAAATGGTGCCGAAACAATCAAT
         SD        (M) A  E  L  T  K  I  T  R  G  M  Q  N  G  A  E  T  I  N
145 GATAATTTAAACAAACTGAACACCATTACTGTTCAGAAAACTGGGGATGAAACAATTGCAGGAAAGAAAACT
     D  N  L  N  K  L  N  T  I  T  V  Q  K  T  G  D  E  T  I  A  G  K  K  T
217 TTCTCTGGTGACGTTAGTGTAGATGGTGATTTCACGATGAAAAAATTTGCGGATTCTTATGTCGCCTTTTTT
     F  S  G  D  V  S  V  D  G  D  F  T  M  K  K  F  A  D  S  Y  V  A  F  F
289 GCAAATAAAGGTAGTGGAAATACAGTCACATTTACTGCACCTTGGGACTGTACTGCAGAAGTTGAACTCTTT
     A  N  K  G  S  G  N  T  V  T  F  T  A  P  W  D  C  T  A  E  V  E  L  F
361 TATCATGGCTGGGGATATAGTGGTGGAGAATGGGAAATCGGAATTACTACTCCCTCCGGATTAACTCAGATT
     Y  H  G  W  G  Y  S  G  G  E  W  E  I  G  I  T  T  P  S  G  L  T  Q  I
433 TATGAAGCCACAGGATATACTAATGGTCACGATAACCAAGCTATATCCATGCCTACAAAGGCAATCTACTCC
     Y  E  A  T  G  Y  T  N  G  H  D  N  Q  A  I  S  M  P  T  K  A  I  Y  S
505 GGTCTCAAAAAAGGGCAACAATACACCTTTGATAAACGTGATGCAAGCGGAAGAGGCGGGGGCCCAAAACAC
     G  L  K  K  G  Q  Q  Y  T  F  D  K  R  D  A  S  G  R  G  G  G  P  K  H
577 CCAATGATGATTGTAAAACTTTATCGGAATTAG
     P  M  M  I  V  K  L  Y  R  N  -
```

FIG. 8. Nucleotide sequence of the phage Tuc2009 *mp1* region. *mp1* runs from nucleotides 88 to 609. The inferred amino acid sequence is given below the DNA sequence. The N-terminal methionine residue (M) is not present in the final protein, as determined by N-terminal protein sequencing. Putative −35 and −10 promoter sequences, as well as a SD sequence and the start codon, are underlined. GS data base accession number L31365.

```
   1 ATGAACAAGAATATGCAAAATCTTGCTCCAGTTGACACAGGTAATATGAAACGTTCAATAACCAGTGAATTT
                                     -35                      -10
  73 ACAGACGGGGGTCTTTCAGGAACGACTGGACCTCATACTGATTATGCTGGATATGTAGAGTATGGGACGCGA

 145 TTTCAATCTGCACAACCATTTGTAAAACCTGCGTTTAACATTCAGAAAAAAGTATTCACAAATGATTTAGAA
                                        -35                      -10
 217 AGGTTGACGAAATGATTAAAACTCGAGACCAATCTATTTTTGATGAATTGTTCAAACGAATACAAGCTTTGG
      SD         M  I  K  T  R  D  Q  S  I  F  D  E  L  F  K  R  I  Q  A  L
 289 GATATACCGTTTATGATTATAAGCCAATGAATGAAGTAGGCTATCCATTTGTTGAATTGGAGAATACTCAAA
     G  Y  T  V  Y  D  Y  K  P  M  N  E  V  G  Y  P  F  V  E  L  E  N  T  Q
 361 CTATTCATGAAGCAAATAAAACGGATATTAAAGGCACAGTAAGTCTTTCATTATCTGTTTGGGGCTTACAGA
     T  I  H  E  A  N  K  T  D  I  K  G  T  V  S  L  S  L  S  V  W  G  L  Q
 433 AGAAGCGCAAGGAAGTATCTGATATGGCAAGCAATATATTTAATCAAGCATTGAATATAAGTGCCACAGATG
     K  K  R  K  E  V  S  D  M  A  S  N  I  F  N  Q  A  L  N  I  S  A  T  D
 505 GCTATTCTTGGGCTTTGAATTCACAAGCAAGTACCATTCAAATGCTGGACGATACAACAACACATACACCTC
     G  Y  S  W  A  L  N  S  Q  A  S  T  I  Q  M  L  D  D  T  T  T  H  T  P
 577 TTAAAAGAGCGTTGATTAACTTAGAATTTAGACTAAGATAGGAGATTTAATATGGCAGAATTAACAGCCAAA
     L  K  R  A  L  I  N  L  E  F  R  L  R  - SD        (M) A  E  L  T  A  K
 649 CAGGGTAAAGATATTATCTTGCTCTATCGTTTGCTTAGTAAAGCAACAAAAGAAGCCGCTTGGAAACTTGCA
      Q  G  K  D  I  I  L  L  Y  R  L  L  S  K  A  T  K  E  A  A  W  K  L  A
 721 TTCCAAACAGAACACTCGAATGAAAAAACTCGAGATTACAACACTACAGCTACCAAAGATGGGACAATAGGT
      F  Q  T  E  H  S  N  E  K  T  R  D  Y  N  T  T  A  T  K  D  G  T  I  G
 793 TCTCTTGCAGCAATTGAATACAGTTTGTCTGCCACATCTATTGCAGCAAATGGTGACCCACATCTTGACGAA
      S  L  A  A  I  E  Y  S  L  S  A  T  S  I  A  A  N  G  D  P  H  L  D  E
 865 ATGGACAAAGCGTTTGATGATGGAGAAATTATTGACGTGTGGGAAATTGATAAAGCTGAAAAAGGATCTGAC
      M  D  K  A  F  D  D  G  E  I  I  D  V  W  E  I  D  K  A  E  K  G  S  D
 937 GGAAAGTACAAAGCTAAATATCTTCGTGCTTATCTTACAAGTTTCTCTTATGAACCTAACTCAGAAGATGCG
      G  K  Y  K  A  K  Y  L  R  A  Y  L  T  S  F  S  Y  E  P  N  S  E  D  A
1009 CTTGAATTGAGTTTAGAATTTGGAGTGTTTGGTAAACCTCAAAAGGGCCAAGCTACACTAACTGAAGAACAA
      L  E  L  S  L  E  F  G  V  F  G  K  P  Q  K  G  Q  A  T  L  T  E  E  Q
1081 GCTAATGTTGTTCAGTATGTCTTCAAAGATACTGTTGCGGGATAAAGCTGAAAATATTACTGACTCTGCCTG
      A  N  V  V  Q  Y  V  F  K  D  T  V  A  G  -
1153 GAGTACAGTTGTAGAAGTGACAATTTAAATACTATAAACAAAAGGCTAGAGATTTGCTCTATTCTTTATTTT
```

FIG. 9. Nucleotide sequence of the phage Tuc2009 *mp2* region. *mp2* runs from nucleotides 628 to 1125. ORF *x* runs from nucleotides 228 to 617. The inferred amino acid sequences are given below the DNA sequence. The N-terminal methionine residue (M) in the inferred MP2 sequence is not present in the final protein, as determined by N-terminal protein sequencing. Putative −35 and −10 promoter sequences, as well as SD sequences and start codons, are underlined. The putative transcription terminator downstream of *mp2* is doubly underlined. GS data base accession number L31366.

phage head is initiated. Packaging by this mechanism gives rise to a set of circularly permuted molecules, each larger than the unit genome size, with terminal redundancy. The presence of overlapping submolar fragments in *Eco*RV and *Pvu*II digests of the phage DNA enabled us to locate the *pac* site on the genomic map and to determine the direction of packaging (Fig. 2). The attachment site (*attP*) and the integrase gene (*int*) appeared to be located at the part of the map opposite *pac*, as is the putative repressor gene *c*I. These determinants will be described in more detail elsewhere (29, 30).

The sequences that encode the lysin and a putative holin, *lys* and *S*, respectively, were identified and, as found in analogous systems, appear to be part of one operon in which *S* precedes and partially overlaps *lys*. The presumptive holin shares several structural characteristics with other known holins (36). At the DNA level, Tuc2009 shows the same organization of its *S* and *lys* genes as that of the lambdoid phages λ, 21, PA-2 (*E. coli*), and P22 (*Salmonella typhimurium*), i.e., *S* precedes *lys* and both ORFs overlap (by 4 bp in the case of Tuc2009) (Fig. 3). At the protein level, the size of the Tuc2009 *S* product (88 amino acid residues; predicted molecular weight, 9,700) agrees well with the average holin size of approximately 70 to 110 residues. Furthermore, as in other holins, two strongly hydrophobic potential transmembrane domains can be recognized as well as a highly charged C-terminal domain. A dual-start motif, a feature conserved in many but not all holin genes, does not seem to be present in the Tuc2009 gene. When present, this motif allows the formation of two gene products with opposing effects, starting at either Met-1 or a Met residue further downstream (usually at position 3 or 4). In Tuc2009, the fourth codon of the *S* gene reads ATC rather than ATG (confirmed by direct DNA sequencing of the phage genome). The absence of a dual-start motif, which in other bacteriophages plays an important role in controlling holin activity, raises the question as to how holin expression and/or activity is regulated in Tuc2009. The holin-coding sequence is preceded by a consensus SD sequence and several putative promoters, with the one located closest to the coding sequence exhibiting the highest identity to the consensus sequence (32). The distance between this promoter and the coding sequence is very short, however. On the basis of a comparison of *L. lactis* transcription initiation sites (32), transcription using this promoter may start at the beginning of the putative SD sequence indicated in Fig. 3, but one can only speculate whether this transcript would subsequently allow an efficient translation of the holin gene, since in known *L. lactis* translation initiation sites, a stretch of A and U residues usually precedes the SD sequence (32). The transcript may be functional mainly in expression of the downstream lysin gene. In that way this promoter may allow the differential regulation of holin and lysin expression. In addition, one expects holin and lysin expression to be affected by repressor action. Although a repressor-coding sequence has been identified in Tuc2009 (30), so far no data on repressor binding and the consequent effect on gene expression are available.

The manner in which *S* and *lys* are organized, with the stop codon of *S* overlapping the start codon of *lys*, resembles a configuration which of several configurations tested, was found to be the most effective in establishing transitional coupling between two ORFs in *L. lactis* (31, 33). Additional research is needed to confirm translational coupling between *S* and *lys* and to clarify the significance of the different configurations found in φUS3 and mv1, where the proposed holin and lysin genes are further apart.

While in the lambdoid phages, 21, PA-2, and P22, the lysis cassette contains a third gene, *Rz*, downstream of the lysin gene (36), an analogous ORF cannot be found in Tuc2009. In fact, a strong transcriptional terminator appears to be present immediately downstream and overlapping the 3′ end of the lysin gene. In bacteriophage EJ-1, a third ORF with unknown function (10), for which no analog can be found in Tuc2009 either, is present upstream of the presumptive holin gene. While the putative holins of Tuc2009 and EJ-1 are very similar, their lysins show little or no homology. In contrast, the bacteriophage Cp-7 lysin shows considerable homology to the Tuc2009 lysin, but the protein encoded by the ORF preceding the lysin gene in this phage shows little homology to the Tuc2009 holin, if it encodes a holin at all. Therefore, it seems that individual genes, rather than the whole lysis cassette, are conserved, an observation also made for lambdoid phages (36).

SDS-PAGE analysis showed that the most abundant phage protein, which is likely to be the major capsid protein, had a molecular weight of 32,000. A similar size has been reported for the major capsid proteins of the *L. lactis* bacteriophage F4-1 (8, 14) and the *Lactobacillus delbrueckii* bacteriophages LL-H and mv4 (34). The molecular weights of some other Tuc2009 structural proteins for which the N-terminal amino acid sequences have been determined were estimated at 29,000, 22,000 (or based on DNA sequence data, 18,100), and 19,000, respectively. The ORFs encoding the latter two proteins, *mp2* and *mp1*, respectively were identified and localized on the genomic map, where they appeared to be 9 kb apart. The ORFs encoding the larger two proteins did not appear to be present in the intervening region or in a 3-kb region directly upstream of *mp2*, which comprises the *pac* site, or in a 7-kb region directly downstream of *mp1*, which extends beyond the lysin-coding region.

The proteins, as deduced from the *mp1*, *mp2*, and ORF *x* DNA sequences, did not show any significant homology to data base sequences (SWISS-PROT, PIR, and GenPept-translated GenBank sequences). Because information on structural proteins of lactococcal bacteriophages is only starting to become available, future research will undoubtedly reveal similarities among these proteins which are already known to exist among other phage-encoded proteins (reference 29 and unpublished observations).

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