Genetic and Biochemical Characterization of the *Lactobacillus delbrueckii* subsp. *lactis* Bacteriophage LL-H Lysin

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LL-H, a virulent phage of *Lactobacillus delbrueckii* subsp. *lactis*, produces a peptidoglycan-degrading enzyme, Mur, that is effective on *L. delbrueckii*, *Lactobacillus acidophilus*, *Lactobacillus helveticus*, and *Pediococcus damnosus* cell walls. In this study, the LL-H gene *mur* was cloned into *Escherichia coli*, its nucleotide sequence was determined, and the enzyme produced in *E. coli* was purified and biochemically characterized. Mur was purified 112-fold by means of ammonium sulfate precipitation and cation-exchange chromatography. The cell wall-hydrolyzing activity was found to be associated with a 34-kDa protein. The C-terminal domain of Mur is not essential for catalytic activity since it can be removed without destroying the lytic activity. The N-terminal sequence of the purified lysin was identical to that deduced from the nucleotide sequence, but the first methionine is absent from the mature protein. The N-terminal part of this 297-amino-acid protein had homology with several *Chalaropsis*-type lysozymes. Reduction of purified and Mur-digested *L. delbrueckii* cell wall material with labeled NaB³H₄ indicated that the enzyme is a muramidase. The temperature optimum of purified Mur is between 30 and 40°C, and the pH optimum is around 5.0. The LL-H lysin Mur is stable at temperatures below 60°C.

The last step of phage infection is the release of mature phage particles by lysis of the host cell. Phage lysins are enzymes capable of degrading bacterial cell wall peptidoglycan. In the case of gram-positive bacteria, lysis by autolytic enzymes can be readily induced by changing the physiological conditions, and the role of bacterial autolysins in cell lysis during phage infection has been suggested (50). However, several lysins produced by phages infecting gram-positive bacteria have been described (see reference 66 and references therein).

On the basis of the bond specificity, four common types of phage lysins are recognized. (i) Lysozyme-like enzymes (muramidases) hydrolyze *N*-acetylmuramyl-1,4- β -*N*-acetylglucosamine bonds (4, 7, 9, 13, 24–26, 47, 51, 64). The coliphage T4 gene *e* lysozyme (61) is a well studied representative of this group. Muramidases can be divided further into four homology groups on the basis of amino acid sequence, namely, chicken egg type lysozymes, goose type lysozymes, phage type lysozymes, and fungal type lysozymes (64). (ii) Lytic transglycosylases, as an example, the *R* gene product of the coliphage lambda (6), act on the same bond as muramidases but further catalyze the intramolecular transfer of the *O*-muramyl residue to its own C-6 hydroxyl group. (iii) *N*-Acetylmuramyl-L-alanine amidases hydrolyze the bond between *N*-acetylmuramic acid and L-alanine (38, 49). The amidase encoded by the gene *15* of coliphage T7 (31) represents this type of phage lysins. (iv) Peptidases, for example, the peptidase produced by the enveloped RNA-containing phage ϕ 6 of *Pseudomonas phaseolicola* (12), hydrolyze the peptidoglycan stem or bridge peptides.

The peptidoglycan layer of gram-positive bacteria is much thicker than that of gram-negative bacteria. Teichoic acids, lipoteichoic acids, and glycolipids are characteristic components of the cell wall-membrane complex in a large group of gram-positive bacteria. It has been suggested that bacterial autolytic enzymes (autolysins) play an essential role in cell

growth and cell separation (15). Although peptidoglycan is the substrate being degraded, the enzymes that degrade it may have strict host specificity. The lytic enzymes active on the cell wall of gram-negative bacteria can be ineffective on the cell wall of gram-positive bacteria. Several studies suggest that teichoic acids or lipoteichoic acids may take part in regulation of the activity of the bacterial autolysins (15, 22, 30, 59). Since the compositions of the teichoic acids and lipoteichoic acids are different in the cell walls of different bacterial species, it is difficult to find a lytic enzyme that is efficient on a wide range of gram-positive bacterial species.

In dairy biotechnology, the availability of lysin proteins provides the opportunity to eliminate specific contaminant species and to facilitate the release of intracellular enzymes during and after dairy fermentations. Several phage lysins effective on dairy lactococci have been cloned and sequenced. These include the lysins produced by *Lactococcus lactis* phages vML3, P001, c2, ϕ US3, and Tuc2009 (4, 27, 46, 55, 63). *Lactobacilli* are another economically important group of dairy lactic acid bacteria used in the manufacturing of cheese and yogurt. Among phage lysins of *lactobacilli*, the muramidase produced by *Lactobacillus casei* phage PL-1 has been purified and characterized (29).

Phage LL-H is a virulent phage of *Lactobacillus delbrueckii* subsp. *lactis*, a species widely used as a starter for production of Emmental cheese in Finland (2). Phage LL-H can be viewed as a reference phage because it is the best studied representative of the DNA homology group that contains most of the known *L. delbrueckii* phages (39). Trautwetter et al. (60) have previously shown that a 4.35-kb *Pst*I restriction fragment of LL-H DNA cloned into *Escherichia coli* produces an enzyme that hydrolyzes the cell wall of *L. delbrueckii*. The nucleotide sequence of the lysin gene of *Lactobacillus delbrueckii* subsp. *bulgaricus* phage mv1, which belongs to the same DNA homology group as phage LL-H, has been published (8).

In this paper, we describe the cloning of the phage LL-H lysin gene *mur* into *E. coli*, its nucleotide sequence, the puri-

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fication of the lysin protein Mur in an active form, and its biochemical characterization.

MATERIALS AND METHODS

Bacteria, phages, and vectors. *L. delbrueckii* subsp. *lactis* phage LL-H was originally isolated from a cheese processing plant in Finland in 1972 and was obtained from Valio, Cooperative Dairies' Association, Helsinki, Finland (2). For expression of the phage lysin, the lysin gene *mur* was cloned into the expression vector pTZ18R (41), BlueScriptKS⁺ (Stratagene), or pPLc245 (48). For DNA sequencing, genomic fragments of LL-H DNA were cloned into M13mp18/19 vectors (65). *E. coli* JM101 (65) was used as a host strain. *Propionibacterium freudenreichii* van Niel, *Bacillus subtilis*, *Clostridium tyrobutyricum*, *Clostridium sporogenes*, *Lactobacillus acidophilus*, and *Lactobacillus helveticus* were obtained from the American Type Culture Collection. *L. delbrueckii* subsp. *lactis* LKT, LL23, and LL78 and the *Lactobacillus casei* strain 1/3 were obtained from Valio. *L. delbrueckii* subsp. *bulgaricus* CH-2 and B2 were from CHR Hansen's Laboratorium (Copenhagen, Denmark) and from P. Ritzenthaler (Centre Nationale de la Recherche Scientifique, Toulouse, France) respectively. The *Lactococcus lactis* strain F7/2 was from M. Teuber (Kiel, Germany). The *Pediococcus damnosus* strain E-86274 was from A. Haikara (Valtion Teknillinen Tutkimuskeskus, Espoo, Finland). The *Micrococcus luteus* and *Streptococcus pneumoniae* strains were clinical isolates from the Department of Microbiology, University of Oulu.

Cloning of the gene *mur*. All DNA manipulations were done by standard procedures (52). A 3.1-kb *Hind*III fragment of the phage LL-H DNA was cloned into the pTZ18R vector. The resulting plasmid, pTZ-lys, was transformed into the *E. coli* strain JM101. Transformants were selected on the basis of their resistance to ampicillin. The presence of the gene *mur* was confirmed by restriction enzyme analysis and by the ability of the sonicated bacteria to lyse *L. delbrueckii* cell lawns. For cloning of the open reading frame (ORF) encoding the phage lysin, primers surrounding the gene *mur* and containing *Sall* sites were designed. The primer MurSalA (5'-GCTGATCGTCGACCACTTAAGGC-3') hybridized upstream of *mur*, and the primer MurSalC (5'-CTTGCGCGTCGACAGCTGCACTC-3') hybridized downstream of *mur*. The DNA fragment between these primers was amplified by PCR with *Taq* polymerase (30 cycles of denaturation [1 min, 94°C], annealing [1 min, 46°C], and extension [2 min, 72°C]). The resulting 1.1-kb DNA fragment was digested with *Sall* and ligated with the *Sall*-digested BlueScriptKS⁺ vector (Stratagene). For cloning of the truncated form of *mur* encoding the catalytic domain of the enzyme, the primer MurDel (5'-CGCTGTTGTGATCAACCACTGCC) containing a *Bcl*I restriction site and the primer LysPCR1 (5'-TCTATGTCCACTTGAAG) hybridizing upstream of *mur* were used in PCR (reaction conditions same as those described above). The amplified DNA fragment containing the truncated gene *mur* was digested with *Bcl*I and ligated with vector pTZ18R linearized with *Bam*HI.

DNA sequencing and sequence analysis. The nucleotide sequence was determined in both directions by the method of Sanger et al. (53). DNA fragments cloned into M13mp18/19 vectors were sequenced with Sequenase version 2.0 (United States Biochemical). The cycle sequencing technique modified from the method of Murray (43) and TASequence version 2.0 (United States Biochemical) were used to confirm the nucleotide sequence with direct sequencing from the phage LL-H DNA. Computer analyses were performed by use of the Genetics Computer Group sequence analysis software package version 7.2-UNIX (20), LKB DNASIS (V7.0) and LKB PROSIS (V6.02). Database searches were performed with the programs FASTA (45) and BLAST (3).

Purification of the cloned gene *mur* product. The *E. coli* JM101 cells containing the pTZ-lys plasmid were grown overnight in 500 ml of Luria-Bertani (LB) medium containing 50 µg of ampicillin per ml and 5 mM glucose at 37°C. Bacterial cells were collected by centrifugation (10,000 × g, 5 min), resuspended in 6 ml of 20 mM sodium phosphate (pH 6.0), containing 1 mM phenylmethylsulfonyl fluoride, and 2 mg of streptomycin sulfate was added to precipitate ribosomal proteins. Bacterial cells were sonicated (MSE Soniprep 150 apparatus, 25-µm amplitude, five bursts of 20 s), and the cell debris was removed by centrifugation (10,000 × g, 10 min). Proteins were precipitated with ammonium sulfate (60% saturation), and the mixture was kept in ice for 15 min and centrifuged (10,000 × g, 15 min). The protein precipitate was dissolved in 2 ml of loading buffer (50 mM sodium phosphate [pH 6.5], 0.25 M sodium chloride), and the sample was dialyzed against the same buffer overnight. The dialyzed sample was incubated again at 4°C overnight, and all insoluble material was removed by centrifugation (10,000 × g, 25 min). The sample was then applied to a carboxymethyl (CM)-Sephadex C-50 cation-exchange column (Pharmacia; 2 by 7 cm; bed volume, 14 ml) previously equilibrated with the loading buffer. The column was washed with 100 ml of loading buffer at a flow rate of 50 ml/h. Proteins were eluted with a linear gradient of 0.2 to 0.8 M sodium chloride in 50 mM sodium phosphate (pH 6.5), at the same flow rate. Fractions containing lytic activity were pooled and concentrated by dialysis against dry polyethylene glycol 20000. Lytic activity was monitored on agar plates, as described by Boizet et al. (8).

Electrophoresis, electroblotting, and N-terminal amino acid analysis of the LL-H lysin. Protein samples were run on a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel (34) and stained with Coomassie brilliant blue R-250. For N-terminal amino acid sequence analysis, proteins were electrotransferred from

the gel onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore) in CAPS [3-(cyclohexylamino)-1-propanesulfonic acid] buffer, as described by Matsudaira (40). Proteins were stained with Coomassie brilliant blue R-250, cut off from the membrane, and sequenced on an Applied Biosystems 477A sequencer (Protein Research Unit, Turku Centre for Biotechnology), as described by Bauermann (5).

Detection of lytic activity by SDS-polyacrylamide gel electrophoresis. Enzymatic activity was detected in situ in an SDS-12% polyacrylamide gel containing *L. delbrueckii* cells, essentially as described by Leclerc and Asselin (35). Bacterial cells from an overnight culture (30 ml) were harvested by centrifugation (10,000 × g, 5 min, 4°C), suspended in 4% SDS, and boiled for 10 min to inactivate the bacterial autolysins. After centrifugation (10,000 × g, room temperature [RT]), the pellet was washed once with distilled water, collected by centrifugation, and suspended in 2 ml of water. The cell suspension (2 ml) was mixed with the other components of the 12% separating gel solution (total volume, 30 ml) before gel casting and polymerization (34). SDS and sucrose (final concentrations, 2.5 and 1%) were added to the protein samples, but the samples were not heated prior to electrophoresis. After electrophoresis, the gel was incubated for 24 h at 37°C in 125 ml of 25 mM Tris-HCl (pH 7.5) containing 1% Triton X-100 to permit protein renaturation. Transparent bands were visible in the translucent gel where cell wall-hydrolyzing activity was present. The gel was photographed against a dark background.

Isolation and purification of *L. delbrueckii* cell walls. The enzymatic specificity of Mur was determined with purified cell wall material. *L. delbrueckii* subsp. *lactis* LKT cells were grown and harvested as described above. To inactivate the bacterial autolysins, the cell pellet was suspended in a 4% SDS solution and incubated in a boiling water bath for 15 min. The cell suspension was then sonicated (two bursts of 5 min, 30-µm amplitude, RT), unbroken cells were removed by centrifugation (3,000 × g, 10 min, RT), and the cell wall material was collected from the supernatant (20,000 × g, 30 min). The cell wall pellet was washed twice with 50 mM Tris-HCl (pH 7.5) and resuspended in the same buffer. Cell walls were incubated with proteinase K (0.1 mg/ml) at 56°C for 30 min. The suspension was boiled for 10 min in 1% SDS, and the cell walls were collected by centrifugation as described before. The pellet was washed once with distilled water, resuspended in 10% trichloroacetic acid, and incubated at 60°C for 15 min. After centrifugation, the cell wall pellet was washed three times with distilled water, lyophilized, and stored at -20°C.

Determination of the enzymatic activity of the LL-H lysin. Species specificity of the LL-H lysin Mur was tested on agar plates (8) and turbidimetrically. The bacterial strains used for the species specificity analysis were grown to the exponential phase, harvested by centrifugation (10,000 × g, 10 min), and resuspended in lysin buffer (50 mM sodium phosphate [pH 6.0], 100 mM NaCl). Bacterial cells were autoclaved (120°C, 15 min), cooled, collected by centrifugation (10,000 × g, 10 min), and diluted in lysin buffer to yield an A_{600} of about 0.6. Five microliters of an appropriate dilution of the lysin was added to 2 ml of the cell suspension, and the decrease in absorbance was measured. The decrease in A_{600} was measured in a double-beam Beckman model 25 spectrophotometer. The reaction rate ($\Delta A_{600}/\text{min}$) was determined from the linear part of the curve and used to express the activity of the enzyme. The unit activity of the LL-H lysin was expressed as the amount of enzyme that produces a decrease in absorbance of 0.001 unit per min at RT (23°C). The optimal pH was determined with 50 mM sodium phosphate buffer (pH 4.5 to 8.5) or 50 mM sodium citrate buffer (pH range, 4.0 to 6.0). The effects of different salts were studied in sodium phosphate buffer (50 mM sodium phosphate [pH 6.0], 100 mM NaCl). When testing the effects of calcium and zinc, sodium acetate buffer (50 mM sodium acetate [pH 6.0], 100 mM NaCl) was used instead, because of the higher solubilities of Ca and Zn under these conditions. The temperature optimum was determined by measuring the decrease in absorbance at the given temperature in sodium phosphate buffer (50 mM sodium phosphate [pH 6.0], 100 mM NaCl). To determine the thermostability, the enzyme was incubated for 30 min at the desired temperature, and the residual activity was measured at 37°C.

Determination of the enzymatic specificity of the LL-H lysin. Samples containing 500 µg of the purified cell wall material described above and 25 µg of the purified LL-H lysin, in 250 µl of 50 mM sodium phosphate buffer (pH 5.5) containing 200 mM NaCl and 10 mM MgCl₂, were incubated for 20 h at RT. The generation of free reducing groups and amino groups was monitored as described previously (28, 56). The characterization of the reducing groups liberated by the enzyme was carried out essentially as described by Ward (62). The samples were reduced with NaB³H₄ (500 mCi/mmol; Amersham) for 24 h at 4°C, hydrolyzed with 4 M HCl at 110°C for 6 h, and chromatographed on a Dowex 50WX4 column (200/400 mesh, 1 by 20 cm; Bio-Rad). [³H]muramitol and [³H]glucosaminol were prepared by reducing muramic acid and galactosamine obtained from Sigma. Controls of glycosidase activity were performed with samples containing lysozyme in sodium phosphate buffer (pH 7.0), containing 200 mM NaCl, which were treated as described above. Samples containing either buffer, lysin, or undigested cell walls served as controls.

Nucleotide sequence accession number. Nucleotide sequence data have been deposited with GenBank under the accession number M96254.

FIG. 1. Nucleotide sequence of the lysin gene *mur* of phage LL-H. Nucleotide numbers correspond to the numbers in the GenBank sequence file (accession number M96254). The locations of the primers used for amplifying *mur* or the truncated form of it are underlined with a dashed line, and the altered nucleotides are shown above the sequence. The recognition sites of *SalI* (▽) or *BclI* (▼) used for cloning of *mur* or its truncated form, respectively, are shown. The proposed RBS sequences of the ORFs are underlined with a solid line. The horizontal arrows represent the inverted repeat of the hairpin structure Hp.

RESULTS

Cloning and expression of the LL-H lysin in *E. coli*. The LL-H lysin gene designated *mur* hereafter has previously been located in the 4.35-kb *PstI-EcoRI* restriction fragment of the LL-H DNA (60). Further subcloning and expression-detection of activity revealed that the information for the lysis activity lies within the 3.1-kb *HindIII* restriction fragment (data not shown). That 3.1-kb LL-H DNA fragment was cloned into the pTZ18R vector and transformed into the *E. coli* strain JM101. The clone construct, designated pTZ-lys, was used for the expression of the LL-H lysin protein purified and characterized in this study. By amplifying a 1.1-kb DNA fragment containing the 894-nucleotide (*nt*) gene *mur* by PCR (the locations of the oligonucleotides used are shown in Fig. 1) and cloning it into BluescriptSK vector, we also obtained a lysin-expressing clone, designated BS-*mur*. Both pTZ-lys and BS-*mur* clones produced LL-H lysin even without induction of the *lacZ* promoter by IPTG (isopropyl-β-D-thiogalactopyranoside), and the clones easily lost the lytic activity. Therefore, the *E. coli* clones were always grown from a single colony, and the cultures showing lytic activity were gradually scaled to bigger volumes. Since the *lac* operon of the expression vectors pTZ18R and BluescriptSK is not efficiently repressed, we further cloned the 1.1-kb DNA fragment into the vector pPLc245 (pPLc245-*mur*) containing the lambda promoter, which is efficiently repressed by the lambda repressor protein in the *E. coli* host strain K12ΔH1Δtrp. The clone pPLc245-*mur* was more stable than the other clone constructs (data not shown).

Nucleotide sequence of *mur*. Seven ORFs (ORF98, ORF53, ORF140, ORF112A, ORF107, *mur*, and ORF109A) could be found within the sequence between *HindIII* and *AhaIII* restriction sites (GenBank accession number M96254). The 3'-terminal part of ORF112A, the whole genes ORF107 and *mur*, and the 5'-terminal part of ORF109A are presented in Fig. 1. The 894-nt gene *mur* encoded a protein, Mur, with a molecular mass of 32.5 kDa as deduced from the DNA sequence. *mur* was preceded by an ORF (ORF107) of unknown function. These ORFs overlapped by 8 nt. A hairpin structure (Fig. 1, Hp) with a calculated free energy of -17 kcal/mol (ca. -71 kJ/mol) (58) lay immediately upstream of ORF107. This hairpin structure was preceded by the A+T-rich sequence AATAATTATATTA and was followed by five thymidine residues. The hairpin structure Hp and the putative ribosome binding site (RBS) of ORF107 were located inside ORF112A, ORF112A and ORF107 overlapped by 11 nt. The overlapping of the pairs ORF53-ORF140, ORF140-ORF112A, ORF112A-ORF107, and ORF107-*mur* suggests that translational coupling may be involved in the expression of these genes.

The suggested RBSs of ORF53 (AUAGGAGG), ORF140 (AAAGGAG), ORF107 (AGAAAGAAG), *mur* (GGAAGG AAG), and ORF109A (AUAGGAG) were homologous to the RBS consensus sequence uaGAAAGGAGg of phage LL-H (42). ORF112A did not contain a putative RBS, but since it overlapped with ORF140, translational coupling seems possible.

Deletion analysis of the gene *mur*. Boizet et al. (8) have previously cloned the endolysin gene *lysA* of the *L. delbrueckii* subsp. *bulgaricus* phage mv1, a close relative of phage LL-H. They suggested that the gene *lysA* of mv1 is 585 nt in size and encodes a 21-kDa protein. Consequently, a clone, from which the last 278 nt of the phage LL-H gene *mur* had been removed, was constructed (Fig. 1). This clone still displayed cell wall-hydrolyzing activity (data not shown), indicating that the lytic activity lies in the N-terminal part of the phage LL-H protein Mur.

Sequence homology and amino acid sequence analysis. The ORFs ORF53, ORF140, ORF112A, and ORF107 (GenBank accession number M96254) did not show any significant homology with known sequences, except with the corresponding DNA sequences of *L. delbrueckii* subsp. *bulgaricus* phages mv1 (8) and mv4 (21). The DNAs of these *L. delbrueckii* phages were very homologous. However, the proteins encoded by genes ORF107 of LL-H (this study) and *lysB* of mv1 (see reference 66) had different sizes (107 amino acids [aa] versus 124 aa) and structures. They were homologous at their N-terminal ends (81% identity in a 59-aa overlap), but their C-terminal ends diverged totally. In the case of LL-H, ORF107 overlapped with the muramidase-encoding gene *mur*, but the corresponding ORFs of mv1 or mv4 did not.

The lysin of LL-H had homology with several muramidase enzymes, as judged by FASTA (45) or BLAST (3) search. These proteins include the endolysins of *L. delbrueckii* subsp. *bulgaricus* phages mv1 (72% identity in a 202-aa overlap [8]) and mv4 (84% identity in a 297-aa overlap [21]) and the muramidases of the *Streptococcus pneumoniae* phages Cp-1 (CPL-1, 34% identity in a 218-aa overlap [24]), Cp-7 (CPL-7, 36% identity in a 185-aa overlap [26]), and Cp-9 (CPL-9, 35% identity in a 218-aa overlap [26]). The lysins of *Lactococcus lactis* phages LC3 (7) and Tuc2009 (4) had 29% identity in 221- and 218-aa overlaps, respectively. The *N,O*-diacetylmuramidase of the fungus *Chalaropsis* sp. (23) showed 28% identity in a 178-aa overlap, the *N*-acetylmuramidase M1 of *Streptomyces globisporus* (37) showed 24.9% identity in a 209-aa overlap, and the autolytic lysozyme encoded by the *lyc* gene of *Clostridium acetobutylicum* ATCC 824 had 21% identity in a 180-aa overlap (18). No significant homology between the LL-H Mur and the N-terminal sequences of the *L. casei* phage PL-1 *N*-acetylmuramidase (29) and the lysin of *Lactococcus lactis* phage vML3 (55) was found.

The amino acid sequences of the lysin of phage LL-H (this study), the muramidase of the *S. pneumoniae* phage Cp-9 (24), and the lysin of the *Lactococcus lactis* phage Tuc2009 (4) have been aligned in Fig. 2. There was a lysine-rich sequence, KP-KAEAKPKPKPK (amino acids 210 to 224), in the LL-H protein Mur (Fig. 2). This strongly hydrophilic sequence was predicted to be located on the surface of the lysin molecule as analyzed by the method of Kyte and Doolittle (33). Comparison of the amino acid sequence of Mur with those of phages Cp-9 and Tuc2009 (Fig. 2) revealed that the amino acid sequence KP-KAEAKPKPKPK of Mur was located in the region where the homology between these lysins ended and where the cell wall binding sequences of the Cp-9 lysin started (24). In this region, lysine residues are also abundant in the Tuc2009 and the Cp-9 amino acid sequences, but other charged residues (R, D, and E) also exist. Interestingly, a good homology (69% identity and 84% similarity) was found between the lysine-rich sequence of LL-H and the sequence EPKPEPKPEPKPK of the membrane protein M of *Streptococcus equi* W60 (57). The C-terminal domains of LL-H lysin, the lysins of the streptococcal phages Cp-1, Cp-7, and Cp-9, and the lactococcal phage

FIG. 2. Comparison of the lysins of phages LL-H, Cp-9, and Tuc2009. Sequence abbreviations: H, phage LL-H Mur; C, *Streptococcus pneumoniae* phage Cp-9 muramidase CPL-9; T, *Lactococcus lactis* phage Tuc2009 lysin. Pairwise alignments are shown. Identical amino acids (*) and conservative replacements (·) are indicated. The lysine-rich region of Mur has been underlined. Alignment was made with the program Multalin (17). Consens, consensus.

LC3 and Tuc2009 lysins were rich in charged residues, making the C-terminal ends of these lysins very hydrophilic.

Purification of the LL-H lysin. The LL-H lysin Mur was purified from sonicated cells of the *E. coli* clone pTZ-lys by ammonium sulfate precipitation followed by CM-Sephadex C-50 cation-exchange chromatography (Table 1). Mur was liberated from the harvested *E. coli* cells by sonication, and therefore, relatively small sample volumes could be processed. Be-

TABLE 1. Purification of the LL-H lysin Mur

Step ^a	Total activity (U)	Amt of protein (mg)	Sp act (U/mg of protein)	Yield (%)	Purification (fold)
Crude extract (after sonication)	13,700	225	61	100	1
(NH ₄) ₂ SO ₄ precipitate (60% saturation)	10,680	55	194	78	3.2
Cation exchange (CM-Sephadex C-50)	6,300	0.92	6,850	46	112

^a The LL-H lysin was purified from 500-ml overnight cultures of the *E. coli* clone pTZ-lys as described in Materials and Methods.

FIG. 3. Purification of the LL-H lysin Mur and identification of its lytic activity. (A) SDS-PAGE analysis of samples taken at different stages of purification. Lanes: a, crude ammonium sulfate precipitate; b, protein sample eluted from the CM-Sephadex C-50 column; c, ribosomal protein S3 of *E. coli* copurified with Mur when streptomycin sulfate (330 μ g/ml) is not added to the sonicated bacteria to precipitate ribosomal proteins). (B) Detection of Mur activity by SDS-PAGE. The separation gel contained *L. delbrueckii* LKT cells boiled in 4% SDS. After electrophoresis, the proteins were renatured in the presence of Triton X-100 (for details, see Materials and Methods). Lanes: d, sonicated *E. coli* pTZ-lys clone cells; e, ammonium sulfate precipitate of the phage LL-H lysate; f, sonicated *L. delbrueckii* LKT cells. The two bands in lane f represent autolysins of *L. delbrueckii* subsp. *lactis* LKT. Locations of the protein molecular mass standards and the bromophenol blue indicator (BPB) are shown.

cause the calculated pI value of the LL-H lysin is 10.4, Mur could be purified effectively by cation-exchange chromatography under the purification conditions used. Only one other protein bound to and eluted from the chromatography matrix (Fig. 3, lane c). The 26-kDa protein that copurified with Mur was identified as the ribosomal protein S3 of *E. coli* (67) by amino acid sequencing (data not shown). This contaminating protein could be removed by precipitating the bacterial ribosomes with streptomycin sulfate, as indicated in Materials and Methods (Fig. 3, lanes b and c). Mur was purified 112-fold to near homogeneity (Table 1; Fig. 3). The molecular mass of the purified Mur (approximately 34 kDa) (Fig. 3, lane b) corresponded to that of the lytic band observed in an SDS-polyacrylamide gel containing *L. delbrueckii* cells after renaturation of the proteins (Fig. 3, lane d). The determined amino acid sequence SSKTYGVDVAXY of Mur was identical to that deduced from the nucleotide sequence (Fig. 1), except that the N-terminal methionine was absent from the native protein. The unidentified amino acid is most probably serine, as deduced from the sequence of the gene *mur* (see Fig. 1).

Enzymatic specificity of the LL-H lysin. Incubation of cell walls from *L. delbrueckii* LKT with the purified LL-H lysin resulted in a 15-fold increase in the reducing power of the suspension. The effect of hen egg lysozyme was similar. No concomitant increase in the number of free amino groups was observed (data not shown). These results suggested that Mur was a glycosidase. To determine the specificity of this glycosidase, samples of cell walls treated with Mur were reduced with NaB^3H_4 to label the free reducing groups generated. After hydrolysis with HCl and chromatography on Dowex 50, the radioactivity was found mainly as [^3H]muramitol (Fig. 4). Identical results were obtained with control samples of cell walls digested with chicken egg lysozyme (data not shown). In contrast, [^3H]muramitol was not detected in control samples con-

taining untreated cell walls or only the enzyme. These results clearly indicated that Mur was a muramidase.

Bacterial species specificity of the LL-H lysin. The LL-H lysin Mur effectively hydrolyzed the cell walls of *L. delbrueckii* subsp. *lactis*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. helveticus*, and *Pediococcus damnosus* but not of the *L. casei* strain 1/3 (Table 2). Bacterial species present in milk products either as commonly used starters (*Propionibacterium freudenreichii*, *Lactococcus lactis* subsp. *diacetylactis*) or as contaminants (*C. sporogenes*, *C. tyrobutyricum*) were resistant to LL-H

FIG. 4. Analysis of free reducing groups in *L. delbrueckii* cell walls digested by the purified LL-H lysin. Samples of cell walls were incubated with the enzyme, reduced with NaB^3H_4 , hydrolyzed with HCl, and chromatographed on a Dowex 50WX4 column, as described in Materials and Methods. Samples of the eluate fractions (100 μ l) were analyzed for radioactivity. The column chromatography profiles corresponding to digested (—) and undigested (---) cell walls are shown. The numbered arrows indicate the elution positions of acidified NaB^3H_4 (arrow 1), [^3H]muramitol (arrow 2), muramic acid (arrow 3), [^3H]glucosaminol (arrow 4), and glucosamine (arrow 5).

TABLE 2. Bacterial species specificity of the LL-H lysin Mur

Species	Strain(s)	Activity ^a
<i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i>	Three strains	+
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	Two strains	+
<i>Lactobacillus acidophilus</i>	ATCC 4356	+
<i>Lactobacillus helveticus</i>	ATCC 15009	+
<i>Lactobacillus casei</i>	1/3	—
<i>Pediococcus damnosus</i>	E-86274	+(P)
<i>Propionibacterium freundenreichii</i>	ATCC 6207	—
<i>Lactococcus lactis</i> subsp. <i>diacetylactis</i>	F7/2	—
<i>Streptococcus pneumoniae</i>	Clinical isolate	—(T)
<i>Micrococcus luteus</i>	Clinical isolate	—
<i>Bacillus subtilis</i>	ATCC 27370	—
<i>Clostridium sporogenes</i>	ATCC 3584	—
<i>Clostridium tyrobutyricum</i>	ATCC 25755	—
<i>Escherichia coli</i>	B. JM101	—

^a Unless otherwise stated, the species specificity was tested both on agar plates (P) and by turbidimetric analysis (T).

lysin treatment, as were *Micrococcus luteus*, *Streptococcus pneumoniae*, *B. subtilis*, and *E. coli* species (Table 2).

Optimal conditions for Mur activity and stability. The conditions affecting the enzymatic activity of the purified LL-H lysin Mur were studied turbidimetrically with lyophilized *L. delbrueckii* subsp. *lactis* LKT cells as the substrate. The activity of the enzyme at different pH values was studied in both sodium phosphate and sodium citrate buffers. The optimal pH for Mur was around 5.0. Mur was active in a considerably wide temperature range, with an optimum at 30 to 40°C. It was also considerably thermostable, for incubation at 60°C for 30 min decreased the enzyme activity by only 20%. Temperatures above 65°C, however, completely inactivated the enzyme. Interestingly, if lyophilized or viable bacteria were used as a substrate, lysis of bacteria was complete. When autoclaved bacteria were used, only one-third reduction in absorbance ($\Delta A_{600}/\text{min}$) was observed at the end of the experiment (data not shown). This suggests that some additional bacterial enzyme activity is involved in the cell lysis process.

The effects of different cations on the activity of Mur were studied. The enzyme activity under different conditions was compared with the activity measured under standard salt conditions (50 mM sodium phosphate [pH 6.0], 100 mM NaCl). As shown in Table 3, NaCl was found to stimulate the enzyme activity, with a 100% increase of enzymatic activity at 200 mM. Also, 10 mM MgCl_2 doubled the activity, and 10 mM CaCl_2 increased the Mur activity threefold (Table 3). The divalent cation chelating agent EDTA at 10 mM did not abolish enzyme activity. Mercury, zinc, and the commonly used protease inhibitor phenylmethylsulfonyl fluoride had no effect on Mur activity, but cadmium substantially decreased enzyme activity (Table 3). The antibiotics ampicillin and streptomycin used during the expression and purification of LL-H lysin did not decrease the enzymatic activity of Mur (Table 3).

DISCUSSION

DNA sequencing, analysis of lytic activity by SDS-PAGE, and amino acid sequencing of the purified protein showed that the lysin gene *mur* of phage LL-H is 894 nt in size and encodes the 34-kDa lytic protein Mur (Fig. 1 and 3). The gene *mur* overlaps with ORF107. Overlapping of the genes suggests that translational coupling may be involved in expression of these genes. The protein tentatively encoded by ORF107 (107 aa) of phage LL-H had homology (81% identity in a 59-aa overlap) with the protein LysB (124 aa) of phage mv1 (66). Interest-

ingly, LysB has been suggested to be a holin-type lysis protein, although it lacks some typical characteristics (like dual start and two hydrophobic membrane-spanning domains) of phage holins. There is, however, no experimental evidence that the cloned 3.1-kb *HindIII* fragment of LL-H DNA contains a pore-forming holin protein. Since function of the phage holin is essential for the phage-induced bacterial lysis, it is unlikely that such proteins could be so different in otherwise homologous phages. Although the substrate specificity tests (Table 3) suggested that *E. coli* cell walls are not a very good substrate for the LL-H Mur, this enzyme seems to be harmful to *E. coli*. Both the clone carrying the 3.1-kb *HindIII* fragment and the clone carrying the 1.1-kb DNA fragment containing *mur* suffered from instability problems, suggesting that the gene *mur* itself is harmful to *E. coli*.

The LL-H lysin Mur had homology with the lysins of *L. delbrueckii* subsp. *bulgaricus* phages mv1 and mv4, with the lysins of *Streptococcus pneumoniae* phages Cp-1, Cp-7, and Cp-9, and with the lysins of *Lactococcus lactis* phages Tuc2009 and LC3 (Fig. 2). However, the homology between Mur and the other phage lysins (except the lysin of phages mv4) was restricted approximately to the first 200 N-terminal amino acids of these enzymes, while their C-terminal ends were totally different. Deletion analysis of the LL-H gene *mur* showed that the cell wall-hydrolyzing activity of Mur is located in the N-terminal end of the protein. The strong DNA homology between *mur* of LL-H and the corresponding genes of *L. delbrueckii* subsp. *bulgaricus* phages mv1 and mv4 supports the interpretation that the previously reported size of the mv1 *lysA* gene (585 nt) may be due to sequencing errors (8).

The homology between the lysins of lactococcus, lactobacillus, and streptococcus phages suggests a common evolutionary origin of these enzymes. Lactobacilli (e.g., *L. casei*, *L. delbrueckii*, and *L. acidophilus*), lactococci (e.g., *Lactococcus lactis*), and some streptococcal species (*Streptococcus thermophilus*) are widely used in dairy fermentation processes, and they may coexist in milk. According to the theory of modular evolution (10), phages may acquire and change gene modules. Modular design has also been suggested for *Enterococcus hirae* muramidase-2, *Streptococcus faecalis* autolysin (32), and *C.*

TABLE 3. Effect of different conditions on the activity of the LL-H lysin Mur

Substance and concn ^a	Activity (%) ^b
NaCl	
100 mM (control).....	100
200 mM.....	190
500 mM.....	99
MgCl_2 , 10 mM.....	184
CaCl_2 , 10 mM.....	291
CdCl_2 , 1 mM.....	27
$\text{Hg}(\text{NO}_3)_2$, 0.1 mM.....	158
ZnCl_2 , 1 mM.....	100
EDTA, 10 mM.....	136
2-Mercaptoethanol, 1.4 mM.....	96
Triton X-100, 1% (vol/vol).....	136
Polyethylene glycol 6000, 1% (wt/vol).....	110
Phenylmethylsulfonyl fluoride, 1 mM.....	117
Streptomycin, 330 $\mu\text{g/ml}$	127
Ampicillin, 330 $\mu\text{g/ml}$	128

^a PEG, polyethylene glycol; PMSF, phenylmethylsulfonyl fluoride.

^b Activity is expressed as percentages relative to activity under control conditions. The control buffer representing 100% activity was 50 mM sodium phosphate (pH 6.0)–100 mM NaCl, except in the cases of Ca^{2+} and Zn^{2+} . Because these divalent cations were studied in 50 mM sodium acetate (pH 6.0)–100 mM NaCl, this buffer was used as a reference.

acetobutylicum LYC muramidase (19). Although there is no detectable homology between the C domains of the lactobacillus, lactococcus, and streptococcus phage lysins (Fig. 2), there is one common feature. The C-terminal parts of these proteins are strongly hydrophilic. As shown with the phages of *Streptococcus pneumoniae*, phage lysis may have homology with the bacterial autolysin (24). If the C-terminal domain of a phage lysin is involved in binding of the enzyme to the cell wall, a phage may accommodate to a new host by acquiring a genetic element (module) encoding the proper cell wall-binding domain from the new host autolysin gene(s).

By using the weak cation-exchange resin (CM-Sepharose C-50), it was possible to purify the LL-H lysin Mur overproduced in *E. coli* with a proper expression clone (Table 1). The purification of Mur from the phage LL-H lysate was possible but much more complicated than purification of Mur produced in *E. coli*. This was because ammonium sulfate precipitated numerous protein components of the MRS medium together with the LL-H lysin (unpublished results).

Our biochemical analysis (Fig. 4) showed that the LL-H lysin is a muramidase. This is in agreement with the conclusion obtained from comparing sequences. Bacterial cell wall specificity of Mur was restricted to the lactic acid bacterial species that are taxonomically related to *L. delbrueckii* (16, 44), namely, *L. delbrueckii* subsp. *lactis*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. helveticus*, and *Pediococcus damnosus*. On the contrary, the *L. casei* strain 1/3 was not lysed by LL-H lysin treatment (Table 2). When viable or lyophilized *L. delbrueckii* cells were used as the substrate, the LL-H lysin lysed the bacterial cells completely. However, if the bacterial cells collected at the logarithmic growth phase were autoclaved or boiled prior to enzyme digestion, the total amount of reduction in absorbance density was only one-third compared with that of the cases when viable or lyophilized cells were used as the substrate of the LL-H lysin. This suggests that some other enzyme activity besides the LL-H muramidase activity may be involved in the lysis process of *L. delbrueckii* cells. Detection of lytic activity by SDS-PAGE revealed that *L. delbrueckii* subsp. *lactis* LKT contains at least two autolytic enzymes (Fig. 3). It has been suggested that bacterial autolysins play an important role in phage infection. Ronda-Laín et al. (50) have shown that autolysis-defective *Streptococcus pneumoniae* strains are resistant against lysis by phage Dp-1. Thus, the LL-H lysin may also induce the autolytic system of the host cell. However, the possibility that boiling may modify the *L. delbrueckii* cell wall in such a way that Mur cannot completely degrade it cannot be excluded.

The pH optimum for Mur was around 5.0. NaCl (optimal concentration, 200 mM) stabilized and enhanced the activity of Mur. Under these conditions, Mur was considerably thermostable, resisting temperatures of up to 60°C. The enzyme could be incubated at room temperature for at least 10 days without a noticeable loss of activity (data not shown). The divalent cations Ca^{2+} and Mg^{2+} enhanced the activity of Mur, while Cd^{2+} had a deleterious effect (Table 3). Alatosava et al. (1) have previously shown that Ca^{2+} or Mg^{2+} is needed for phage LL-H infection and that Cd^{2+} lowers LL-H infectivity. The addition of the divalent cation chelating agent EDTAIII to remove traces of Ca^{2+} or Mg^{2+} did not suppress the activity of Mur, suggesting that these ions are not essential for muramidase activity. Ca^{2+} and Mg^{2+} ions as well as Cd^{2+} as an inhibitor may, however, act indirectly by binding to the negatively charged components of the cell wall, for example (lipo)-teichoic acids.

Shearman et al. (54) have shown that the autolytic *Lactococcus lactis* strain expressing a lactococcal bacteriophage lysin

gene forms protoplasts at the stationary phase of cell growth. The autolytic activity of this strain has a potential use in early and controlled timing of the lysis of the starter culture. As a result, the intracellular enzymes could be released and flavor formation could be accelerated during cheese manufacturing. Autolysis of *Lactococcus lactis* (14), *L. helveticus* (11), and *Propionibacterium freudenreichii* (36) during cheese manufacturing has been described. Bacterial lysis may also be obtained by the addition of a lytic enzyme during cheese processing. *L. delbrueckii* and *L. helveticus* are widely used in the production of Swiss cheese (Emmental). To date, no effectively lytic enzymes against *L. delbrueckii* have been available. The LL-H lysin could be useful as a cheese-ripening additive. It is specific against *L. delbrueckii* and some related species like *L. helveticus*. It is also very thermostable and may possibly be used throughout the cheese-making processes. The pH optimum of Mur (about 5.0) is near the pH of Swiss cheese (pH 5.5 to 5.7). Because Mur can be produced in *E. coli* and further purified in a simple way, amounts of the LL-H lysin sufficient for cheese manufacturing on an industrial scale could possibly be obtained.

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REFERENCES

- Alatosava, T., T. Juvonen, and R.-L. Huhtinen. 1983. Effect of cadmium on the infection of *Lactobacillus lactis* by bacteriophage LL-H. *J. Gen. Virol.* **64**:1623-1627.
- Alatosava, T., and M. J. Pyhtälä. 1980. Characterization of a new *Lactobacillus lactis* bacteriophage. *IRCS Med. Sci.* **8**:297-298.
- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403-410.
- Arendt, E. K., C. Daly, G. F. Fitzgerald, and M. van de Guchte. 1994. Molecular characterization of lactococcal bacteriophage Tuc2009 and identification and analysis of genes encoding lysin, a putative holin, and two structural proteins. *Appl. Environ. Microbiol.* **60**:1875-1883.
- Baumann, M. 1990. Comparative gas phase and pulsed liquid phase sequencing on a modified Applied Biosystems 477A sequencer. *Anal. Biochem.* **190**:198-208.
- Bienkowska-Szewczyk, K., and A. Taylor. 1980. Murein transglycosylase from phage lambda lysate purification and properties. *Biochim. Biophys. Acta* **615**:489-496.
- Birkeland, N. 1994. Cloning, molecular characterization, and expression of the genes encoding the lytic functions of lactococcal bacteriophage ϕ LC3: a dual lysis system of modular design. *Can. J. Microbiol.* **40**:658-665.
- Boizet, B., Y. Lahbib-Mansais, L. Dupont, P. Ritzenthaler, and M. Mata. 1990. Cloning, expression and sequence analysis of an endolysin-encoding gene of *Lactobacillus bulgaricus* bacteriophage mv1. *Gene* **94**:61-67.
- Bonovich, M. T., and R. Young. 1991. Dual start motif in two lambdaoid S genes unrelated to λ S. *J. Bacteriol.* **173**:2897-2905.
- Botstein, D. 1980. A theory of modular evolution for bacteriophages. *Ann. N. Y. Acad. Sci.* **354**:484-491.
- Bottazzi, V. 1993. Aspects of lactic acid bacteria biology. I. Growth and lysis of *Lactobacillus* cells in cheese, p. 81-97. In A. Zamorani, P. L. Manachini, V. Bottazzi, and S. Coppola (ed.), *Biotechnology and molecular biology of lactic acid bacteria for the improvement of foods and feeds quality*. Istituto Poligrafico e Zeca Dello Stato, Rome.
- Caldentey, J., and D. H. Bamford. 1992. The lytic enzyme of the *Pseudomonas* phage ϕ 6. Purification and biochemical characterization. *Biochim. Biophys. Acta* **1159**:44-50.
- Caldentey, J., A.-L. Hänninen, and D. H. Bamford. 1994. Gene *XV* of bacteriophage PRD1 encodes a lytic enzyme with muramidase activity. *Eur. J. Biochem.* **225**:341-346.
- Chapot-Chartier, M. P., C. Deniel, M. Rousseau, L. Vassal, and J. C. Gripon. 1993. Autolysis of two different strains of *Lactococcus lactis* during cheese ripening. *Int. Dairy J.* **4**:251-269.
- Cleveland, R. F., J.-V. Hölte, A. J. Wicken, A. Tomasz, L. Daneo-Moore, and G. D. Shockman. 1975. Inhibition of bacterial wall lysins by lipoteichoic acids and related compounds. *Biochem. Biophys. Res. Commun.* **67**:1128-1135.
- Collins, M. D., C. Aguirre, J. Farrow, A. Martinez-Murcia, B. Phillips, A. Williams, and S. Wallbanks. 1991. Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse tran-

- scriptase sequencing of 16S rRNA. FEMS Microbiol. Lett. **77**:5–12.
17. Corpet, F. 1988. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. **16**:10881–10890.
 18. Croux, C., and J. L. García. 1991. Sequence of the lyc gene encoding the autolytic lysozyme of *Clostridium acetobutylicum* ATCC824: comparison with other lytic enzymes. Gene **104**:25–31.
 19. Croux, C., C. Ronda, R. López, and J. L. García. 1993. Interchange of functional domains switches enzyme specificity: construction of a chimeric pneumococcal-clostridial cell wall lytic enzyme. Mol. Microbiol. **9**:1019–1025.
 20. Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
 21. Dupont, L., A. Vasala, M. Mikkonen, B. Boizet-Bonhoure, T. Alatossava, and P. Ritzenthaler. Unpublished data.
 22. Fischer, W., P. Rösel, and H. U. Koch. 1981. Effect of alanine ester substitution and other structural features of lipoteichoic acids on their inhibitory activity against autolysins of *Staphylococcus aureus*. J. Bacteriol. **146**:467–475.
 23. Fouche, P. B., and J. H. Hash. 1978. The *N,O*-diacetylmuramidase of *Chaloropsis* species. Identification of aspartyl and glutamyl residues in the active site. J. Biol. Chem. **253**:6787–6793.
 24. García, E., J. L. García, P. García, A. Arrarás, J. M. Sánchez-Puelles, and R. López. 1988. Modular evolution of lytic enzymes of *Streptococcus pneumoniae* and its bacteriophages. Proc. Natl. Acad. Sci. USA **85**:914–918.
 25. García, J. L., A. Arrarás, P. García, C. Ronda, and R. López. 1987. Cloning, purification, and biochemical characterization of the pneumococcal bacteriophage Cp-1 lysin. J. Virol. **61**:2573–2580.
 26. García, J. L., J. M. Sánchez-Puelles, and R. López. 1990. Modular organization of the lytic enzymes of *Streptococcus pneumoniae*. Gene **86**:81–88.
 27. Geis, A. 1992. Cloning and DNA sequence analysis of a lysin gene of the lactococcal bacteriophage P001. Federal Dairy Research Centre, Kiel, Germany.
 28. Ghuysen, J.-M., D. J. Tipper, and J. L. Strominger. 1968. Enzymes that degrade bacterial cell walls. Methods Enzymol. **8**:685–699.
 29. Hayashida, M., K. Watanabe, T. Muramatsu, and M.-A. Goto. 1987. Further characterization of PL-1 phage-associated *N*-acetylmuramidase of *Lactobacillus casei*. J. Gen. Microbiol. **133**:1343–1349.
 30. Höltje, J. V., and A. Tomasz. 1975. Lipoteichoic acid: a specific inhibitor of autolysin activity in *Pneumococcus*. Proc. Natl. Acad. Sci. USA **72**:1690–1694.
 31. Inouye, M., N. Arnheim, and R. Sternglanz. 1973. Bacteriophage T7 lysozyme is an *N*-acetylmuramyl-L-alanine amidase. J. Biol. Chem. **248**:7247–7252.
 32. Joris, B., S. Englebert, C. Chien-Peng, R. Kariyama, L. Daneo-Moore, G. D. Shockman, and J.-M. Ghuysen. 1992. Modular design of the *Enterococcus hirae* muramidase-2 and *Streptococcus faecalis* autolysin. FEMS Microbiol. Lett. **91**:257–274.
 33. Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydrophobic character of a protein. J. Mol. Biol. **157**:105–132.
 34. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
 35. Leclerc, D., and A. Asselin. 1989. Detection of bacterial cell wall hydrolases after denaturing polyacrylamide gel electrophoresis. Can. J. Microbiol. **35**:749–753.
 36. Lemee, R., S. Lortal, B. Cesselin, and J. van Heijenoort. 1994. Involvement of an *N*-acetylglucosaminidase in autolysis of *Propionibacterium freudenreichii* CNRZ 725. Appl. Environ. Microbiol. **60**:4351–4358.
 37. Lichenstein, H. S., A. E. Hastings, K. E. Langley, E. A. Mendiaz, M. F. Rohde, R. Elmore, and M. M. Zukowski. 1990. Cloning and nucleotide sequence of the *N*-acetylmuramidase M1-encoding gene from *Streptomyces globisporus*. Gene **88**:81–86.
 38. Longchamp, P. F., C. Mauel, and D. Karamata. 1994. Lytic enzymes associated with defective prophages of *Bacillus subtilis*: sequencing and characterization of the region comprising the *N*-acetylmuramoyl-L-alanine amidase gene of prophage PBSX. Microbiology **140**:1855–1867.
 39. Mata, M., A. Trautwetter, G. Luthaud, and P. Ritzenthaler. 1986. Thirteen virulent and temperate bacteriophages of *Lactobacillus bulgaricus* and *Lactobacillus lactis* belong to a single DNA homology group. Appl. Environ. Microbiol. **52**:812–818.
 40. Matsudaira, P. 1987. Sequence from picomole quantities of proteins electrophoretically onto polyvinylidene difluoride membranes. J. Biol. Chem. **262**:10035–10038.
 41. Mead, D. A., E. Szczesna-Skorupa, and B. Kemper. 1986. Single-stranded DNA 'blue' T7 promoter plasmids: a versatile tandem promoter system for cloning and protein engineering. Protein Eng. **1**:67–74.
 42. Mikkonen, M., J. Vuoristo, and T. Alatossava. 1994. Ribosome binding site consensus sequence of *Lactobacillus delbrueckii* subsp. *lactis* bacteriophage LL-H. FEMS Microbiol. Lett. **116**:315–320.
 43. Murray, V. 1989. Improved double-stranded DNA sequencing using the linear polymerase chain reaction. Nucleic Acids Res. **17**:8889.
 44. Olsen, G. J., C. R. Woese, and R. Overbeek. 1994. The winds of (evolutionary) change: breathing new life into microbiology. J. Bacteriol. **176**:1–6.
 45. Pearson, W. R., and D. J. Lipman. 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85**:2444–2448.
 46. Platteeuw, C., and W. M. de Vos. 1992. Location, characterization and expression of lytic enzyme-encoding gene, *lytA*, of *Lactococcus lactis* bacteriophage ϕ US3. Gene **118**:115–120.
 47. Rao, G. R. K., and D. P. Burma. 1971. Purification and properties of phage P22-induced lysozyme. J. Biol. Chem. **246**:6474–6479.
 48. Remaut, E., P. Stanssens, and W. Fiers. 1983. Inducible high level synthesis of mature human fibroblast interferon in *Escherichia coli*. Nucleic Acids Res. **11**:4677–4688.
 49. Romero, A., R. López, and P. García. 1990. Characterization of the pneumococcal bacteriophage HB-3 amidase: cloning and expression in *Escherichia coli*. J. Virol. **64**:137–142.
 50. Ronda-Lain, C., R. López, A. Tapia, and A. Tomasz. 1977. Role of the pneumococcal autolysin (murein hydrolase) in the release of progeny bacteriophage and in the phage-induced lysis of the host cells. J. Virol. **21**:366–374.
 51. Saeedi, M. S., K. J. Garvey, and J. Ito. 1987. Cloning and purification of a unique lysozyme produced by *Bacillus* phage ϕ 29. Proc. Natl. Acad. Sci. USA **84**:955–958.
 52. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 53. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
 54. Shearman, C. A., K. Jury, and M. J. Gasson. 1992. Autolytic *Lactococcus lactis* expressing a lactococcal bacteriophage lysin gene. Bio/Technology **10**:196–199.
 55. Shearman, C. A., K. L. Jury, and M. J. Gasson. 1994. Controlled expression and structural organization of a *Lactococcus lactis* bacteriophage lysin encoded by two overlapping genes. Appl. Environ. Microbiol. **60**:3063–3073.
 56. Thompson, J. S., and G. D. Shockman. 1968. A modification of the Park and Johnson reducing sugar determination suitable for the assay of insoluble materials: its application to bacterial cell walls. Anal. Biochem. **22**:260–268.
 57. Timoney, J. F., J. A. Walker, M. Zhou, and J. Ding. Unpublished data.
 58. Tinoco, I., P. N. Borer, B. Dengler, M. D. Levine, O. C. Uhlenbeck, D. M. Crothers, and J. Calla. 1973. Improved estimation of secondary structure in ribonucleic acids. Nature (London) **246**:40–41.
 59. Tomasz, A., and M. Westphal. 1971. Abnormal autolytic enzyme in a *Pneumococcus* with altered teichoic acid composition. Proc. Natl. Acad. Sci. USA **68**:2627–2630.
 60. Trautwetter, A., P. Ritzenthaler, T. Alatossava, and M. Mata-Gilsinger. 1986. Physical and genetic characterization of the genome of *Lactobacillus lactis* bacteriophage LL-H. J. Virol. **59**:551–555.
 61. Tsugita, A., and M. Inouye. 1968. Complete primary structure of phage lysozyme from *Escherichia coli* T4. J. Mol. Biol. **37**:201–212.
 62. Ward, J. B. 1973. The chain length of the glycans in bacterial cell walls. Biochem. J. **133**:395–398.
 63. Ward, L. J., T. P. Beresford, M. W. Lubbers, B. B. Jarvis, and A. W. Jarvis. 1993. Sequence analysis of the lysin gene region of the prolate lactococcal bacteriophage c2. Can. J. Microbiol. **39**:767–774.
 64. Weaver, L. H., D. Rennell, A. R. Poteete, and B. W. Mathews. 1985. Structure of phage P22 gene 19 lysozyme inferred from its homology with phage T4 lysozyme. Implications of lysozyme evolution. J. Mol. Biol. **184**:739–741.
 65. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–108.
 66. Young, R. 1992. Bacteriophage lysis: mechanism and regulation. Microbiol. Rev. **56**:430–481.
 67. Zurawski, G., and S. M. Zurawski. 1985. Structure of the *Escherichia coli* S10 ribosomal protein operon. Nucleic Acids Res. **13**:4521–4526.