CHARACTERIZATION OF *LACTOBACILLUS* BACTERIOPHAGE LL-H GENES AND PROTEINS HAVING BIOTECHNOLOGICAL INTEREST

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Abstract

Two regions of the genome of the *Lactobacillus delbrueckii* subsp. *lactis* bacteriophage LL-H were characterized, representing 14 % of the phage genome. The first region of 2497 bp contained genes encoding phage structural proteins and the second region of 2498 bp genes involved in lytic functions. The nucleotide sequences of the major capsid protein gene *g34*, a putative capsid morphogenesis gene (*ORF178A*), the gene *mur* encoding phage cell wall hydrolase (lysin), the gene *hol* (*ORF107*) encoding the cell membrane permeabilizing phage holin, and six other genes with unknown function were found. Identification of these genes was performed by amino acid sequencing of their encoded proteins (genes *g34* and *mur*), by their physiological effect on *E. coli* (genes *hol* and *mur*), by sequence comparison (genes *mur*, *hol*, *ORF178A*), and by biochemical analysis of their encoded purified protein (gene *mur*). A promoter for the capsid protein encoding gene cluster was determined by primer extension method. A purification method suitable for large scale processing (cation exchange chromatography by expanded bed adsorption method) was developed for the phage LL-H lysin protein Mur. Purified Mur was biochemically determined as a *N*-acetylmuramidase, which was effective on cell walls of *Lb. delbrueckii*, *Lb. helveticus*, *Lb. acidophilus* and *Pediococcus damnosus*. Some biotechnological applications for the lysis genes *hol* and *mur* or the purified protein Mur are suggested. Mur digests *E. coli* cell walls inefficiently, but could still be used for lysis of *E. coli*. Coexpression of the phage LL-H lysin and holin genes yielded to lysis of the *E. coli* host only at low culture densities. Therefore, some chemicals were tested for their ability to trigger lysis of *E. coli* cells overexpressing the phage LL-H gene *mur*. Thymol was found to mimic the physiological effects of the phage holin in a bacterial growth state independent manner. An efficient lysis method utilizing intracellular production of Mur and triggering the lysis with thymol was developed.

Keywords: lactic acid bacteria, lysin, holin, cell wall hydrolase

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Oulu, October 1998 Antti Vasala

Abbreviations

bp	base pair(s)
EBA	expanded bed adsorption
kb	kilobase pair(s) (1000 bp)
kDa	kilodalton(s)
LAB	lactic acid bacteria
Lb.	*Lactobacillus*
Lc.	*Lactococcus*
ORF	open reading frame
PCR	polymerase chain reaction
RBS	ribosome binding site
SDS-PAGE	sodium dodecyl sulphate polyacrylamide-gel electrophoresis
subsp.	subspecies

List of original publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals.

I Vasala A, Dupont L, Baumann M, Ritzenthaler P. & Alatossava T (1993) Molecular comparison of the structural proteins encoding gene clusters of two related *Lactobacillus delbrueckii* bacteriophages. J Virol 67: 3061-3068.

II Vasala A, Välkkilä M, Caldentey J & Alatossava T (1995) Genetic and biochemical characterization of the *Lactobacillus delbrueckii* subsp. *lactis* bacteriophage LL-H lysin. Appl Environ Microbiol 61: 4004-4011.

III Vasala A, Isomäki R, Perttunen J, Myllykoski L & Alatossava T (1998) Purification of *Lactobacillus* phage LL-H muramidase by expanded-bed adsorption chromatography. Recent Res Devel Agric & Biol Chem 2: 387-394.

IV Vasala A, Isomäki R, Perttunen J, Myllykoski L & Alatossava T (1998) Thymol-triggered lysis of *Escherichia coli* expressing *Lactobacillus* phage LL-H muramidase. J Industr Microbiol Biotechnol. Accepted for publication.

Contents

1. Introduction

The group of lactic acid bacteria (LAB) is genetically very divergent. The species of genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus*, *Bifidobacterium*, *Pediococcus*, and *Enterococcus* play an important role in the food industry because of their fermentation capacities. They are widely used as starter strains for manufacturing cheese, yoghurt, sour bread and other fermented food products (vegetables, fish, sausages), as well as for prevention of spoilage of silage (Stiles 1996). In addition, several health benefiting (probiotic) effects have been reported for lactic acid bacteria (especially lactobacilli) colonizing the gastrointestinal tract; e.g. stimulation of immunoglobulin production (Isolauri *et al.* 1995, Link-Amster *et al.* 1994, Perdigón *et al.* 1993), induction of interferon expression in macrophages (Kitazawa *et al.* 1992), acidification of the local environment, production of H_2O_2 (Zheng *et al.* 1994), hypocholesteraemic effects (Fernandes *et al.* 1987), binding of mutagenic compounds (Orrhage *et al.* 1994), production of bacteriocins (Klaenhammer 1993) and prevention of adherence of pathogenic bacteria like *Salmonella typhimurium* and *Neisseria gonorrhoeae* to the epithelial cell (Coconnier *et al.* 1993, Zheng *et al.* 1994). *Lactobacillus delbrueckii* subsp. *lactis*, the host species of bacteriophage LL-H, is used for manufacturing Emmental cheese in Finland. Lactic acid bacteria are GRAS-organisms (Generally Recognized As Safe), and may be therefore better accepted as host bacteria for production of heterologous proteins. Recombinant DNA methods have been exploited for the improvement of existing and the development of new strains of lactic acid bacteria, especially lactococci (McKay & Baldwin 1990). The methodology of genetic engineering has progressed more slowly in lactobacilli compared to lactococci. The progress obtained in genetic engineering of bacteria depends on the availability of genetic engineering tools. For example, methods for the purification and characterization of the intracellular components (bacterial chromosomal and plasmid DNA, RNA and proteins) and transfer of the DNA are required. For still unknown reasons, some lactobacilli, like *Lb. delbrueckii*, contain few or no plasmids (potential start material for construction of cloning vectors) and are poorly transformable. Bacteriophages (viruses infecting bacteria) have been recognized in most species. Bacteriophages have the ability to invade bacterial cells, replicate their genomes, produce new phage particles, exchange DNA with the bacterium (especially temperate phages which can integrate their genome to the host bacterium's chromosome), and lyse

the cell resulting in release of new phage particles. Genetic elements involved in the regulation of the expression of phage genes (promoters, operators, repressors, terminators) may be utilized for construction of cloning and expression vectors for LAB. At the end of the infection most phages lyse their host. Cell lysis is also required for purification of intracellular or periplasmic proteins or nucleic acids from bacteria. Lysis of starter lactic acid bacteria and subsequent release of the intracellular enzymes and aroma forming compounds affect the taste and texture of many dairy products (el Soda 1996). Induced expression of the cloned phage lysis genes in order to obtain bacterial lysis has been studied especially for *Escherichia coli* (Dabora & Cooney 1990) and lactococci (Shearman *et al.* 1994, de Ruyter *et al.* 1997). The bacterial cell wall forms an effective permeability barrier that prevents the transfer of DNA into the cell. Purified cell wall hydrolyzing enzymes may be used to remove or weaken the cell wall, which may enhance transformability of bacterial cells (Chang & Cohen 1979, Shivarova *et al.* 1983, Watanabe *et al.* 1992). Recent research development of utilization of the cloned phage lysis gene's expression to accelerate the ripening processes of dairy products has already been achieved for *Lactococcus lactis* (Sherman *et al.* 1992, Shearman & Gasson 1992, de Ruyter *et al.* 1997).

2. Review of the literature

2.1. General properties of bacteriophages

Frederick Twort in 1915 and Felix d'Herelle in 1917 were the first to recognize viruses which infect bacteria, which d'Herelle called bacteriophages ("eaters of bacteria"). Morphology, the type of nucleic acids, nucleic acid homology, and serology are the most important criteria used in the taxonomy of viruses. Bacteriophages (phages) have been categorized into 12 families and a number of genera (Ackermann 1991, Francki *et al*. 1991). The phage genome may consist of double-stranded DNA, single-stranded DNA, double-stranded RNA or single-stranded RNA. Only a few phages represent cubic, filamentous, or pleomorphic morphology. Tailed phages comprise about 95 % of all phages. The genome of tailed phages consists of a single molecule of double-stranded DNA, but otherwise they form very divergent gropups with different particle sizes, fine structures, protein profiles and life cycles (Ackermann & DuBow 1987, Ackermann *et al*. 1995). They are divided into three large families, the *Myoviridae* (contractile tails), *Siphoviridae* (long, noncontractile tals) and *Podoviridae* (short tails) (Ackermann & DuBow 1987, Ackermann *et al*. 1995).

Phages are intracellular parasites. Bacteria and their phages have a common evolutionary history, and phages may have adapted to their host species by multiple mechanisms (Ackerman & DuBow 1987, Arber 1990, Botstein 1980, Campbell 1988, Díaz *et al*. 1990, DuBose & Hartl 1989, Smith 1989). Nucleic acid homology studies have shown that phages have a chimeric origin. Parts of the viral genome may have been derived from plasmids, transposons, bacteria or even a eukaryotic organism that has been infected by the bacteria (Campbell 1988, Ackermann *et al*. 1995). Based on their life styles, phages can be divided into two groups: the virulent phages capable of only lytic propagation, and the temperate phages capable of either lytic or lysogenic propagation. The virulent life cycle of phages consists of infection of the host, replication of the phage genome, production of phage structural components, phage assembly, and release of the progeny phages. This last step usually, but not always (e.g., filamentous phages f1 and M13 of *E. coli*), involves phage induced lysis of the host cell. In lysogeny the phage genome exists as a prophage resulting in coexistence of phage and

bacterium without lysis. Usually this is achieved by integration of the phage genome into the host bacterium's chromosome by a site-specific integration mechanism (Echols & Guarneros 1983). In some rare cases, the prophage exists as an unintegrated plasmid form (Yarmolinsky & Sternberg 1988). Both the lysogenic and lytic pathways require sophisticated timing of the expression of different gene functions. The genes required for certain purposes and expressed at the same time are located at the same genomic areas (Fig. 1). Typically a phage genome consists of early genes and late genes. Early genes encode products that are needed either for generation and maintenance of lysogeny or for the expression of genes required for the lytic life cycle. These can be, for example, subunits to the host RNA polymerase, phage-encoded RNA polymerase, or antitermination proteins (Roberts 1975, Benedik *et al.* 1983, Brody *et al.* 1983, Dunn & Studier 1983, Daniels *et al.* 1988). More detailed division of early genes to early and delayed early genes have been used in the case of some phages, e.g., phage λ (Blattner & Dahlberg 1972, Roberts 1975, Daniels *et al.* 1988). Genes encoding structural proteins and lysis proteins are typical representatives of late expressing genes. Virulent and temperate *Lactococcus* phages do not share significant sequence similarity. Therefore it has been believed that temperate phages do not contribute significantly to the emergence of new virulent phages (Jarvis 1989, Davidson *et al*. 1990). However, some reports describe sequence similarity between temperate and virulent *Lactobacillus* phages (Mata *et al*. 1986, Lahbib-Mansais *et al*. 1988, Sechaud *et al*. 1988).

2.2. Phage LL-H

LL-H is a virulent phage infecting *Lb. delbrueckii* subsp. *lactis*. It was isolated in 1972 by the dairy company Valio Ltd (previously the Finnish co-operative Dairies' Association) and initially characterized by Alatossava and Pyhtilä (1980). It belongs to the family *Siphoviridae* and has morphological characters typical for group B1 phages. It has a small isometric head and a long noncontractile tail with a baseplate and a tail fiber. Its genome of double-stranded DNA (34.6 kb in size) is circularly permuted and terminally redundant (Forsman & Alatossava 1991). Due to extensive studies in our laboratory (Alatossava 1987, Forsman 1994, Mikkonen 1996) it is now probably the best characterized *Lactobacillus* phage. Beside the present work (Papers I & II), nucleic acid sequence of the phage LL-H genome has been determined and published by Mikkonen and Alatossava (1994), Mikkonen *et al*. (1996) and Mikkonen (1996) (GenBank entries L46882, L37351, L29567, L29658, L42315, and L46883). Like in the coliphage T7 genome, most LL-H genes are transcribed from one strand (Mikkonen, 1996). On the contrast, lysogenic and lytic genes of temperate lactic acid bacteria phages like r1t, Tuc2009, bIL67 and c2 are transcribed from opposite strands (Schouler 1996, van Sinderen *et al.* 1995, van Sinderen *et al.* 1996, Schouler *et al.* 1994, Lubbers *et al*. 1995). By sequence analysis and studies of the temporal transcription, the phage LL-H genome seems to be divided into distinctive areas containing early gene functions (like DNA replication) and late genes (needed for DNA packaging, synthesis of phage structural proteins, and cell lysis) (Fig. 1). The early genes are expressed troughout the infection cycle (Mikkonen *et al*. 1996b). As a transcription control mechanism,

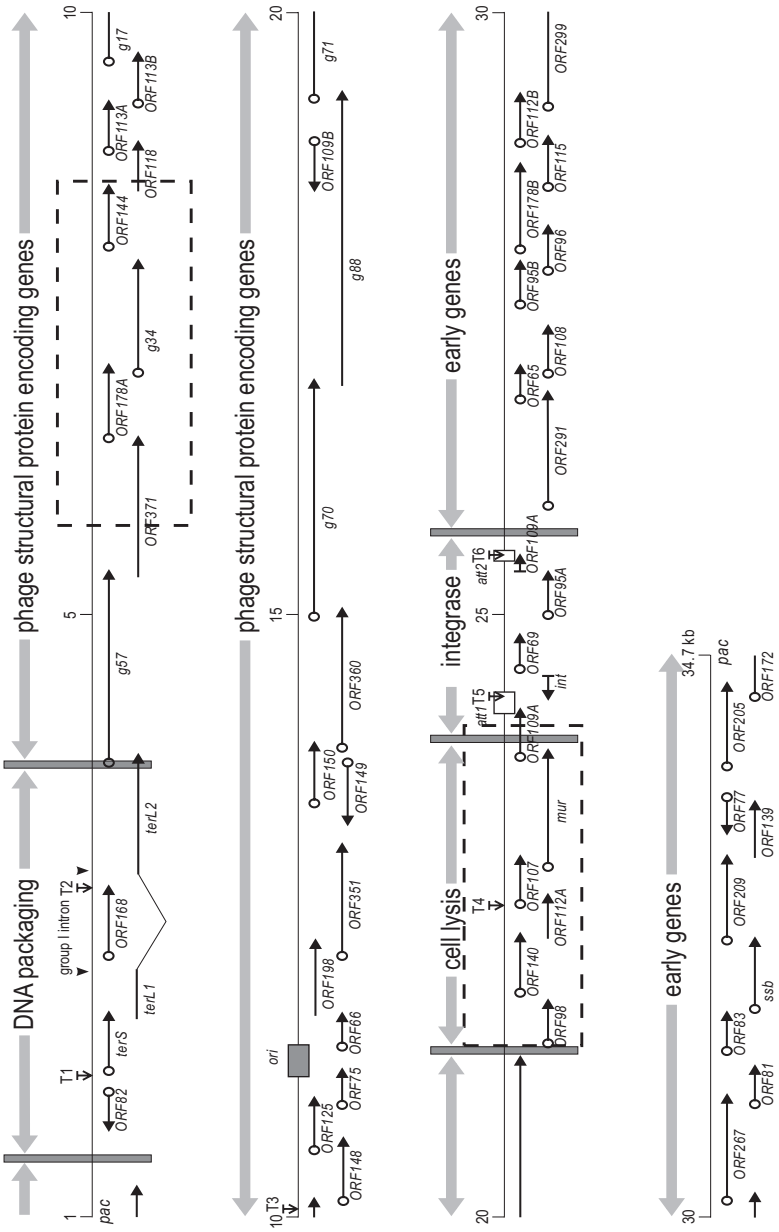

Fig. 1. The genomic organization of phage LL-H (Mikkonen 1996). The horizontal arrows show the directions, positions and lengths of the putative ORFs, with their names given beneath them. An open circle at the beginning of an arrow denotes a ribosome binding site. The small gray box indicates the position of the putative replication origin. The six putative terminators are shown by short vertical arrows (from T1 to T6). The open boxes marked att1 and att2 show the locations and extents of the regions sharing sequence similarity with the phage mv4 attP-site. The genomic areas presented in this thesis are shown by boxes drawn with dashed lines.

Mikkonen (1996) has suggested antitermination mechanism that utilizes phage-encoded antitermination protein, possibly the product of the early gene *ORF267*. A defective integration system in its genome suggests that the ancestor of the phage LL-H has been a temperate phage (Mikkonen *et al*. 1996a). Within the terminase gene (required for DNA packaging), a group I intron has been recognized (Mikkonen & Alatossava 1995). The main coding strand of phage LL-H genome contains 48 putative ORFs, while as the complementary strand has only four short putative *ORF*s. In the classification of *Lb. delbrueckii* phages (Mata *et al*. 1986, Lahbib-Mansais *et al.* 1988), phage LL-H belongs to DNA homology group a. Phages belonging to this homology group exhibit partial genome homology in which highly conserved blocks have abrupt and distinctive boundaries with genome areas not showing any homologies (Alatossava *et al*. 1995). The function of six structural proteins encoding genes, intron encoded endonuclease, and small and large subunits of terminase have been determined. In addition, for 15 ORFs sequence homologies with other organisms have been detected (Mikkonen 1996). Phage LL-H encodes its own murein hydrolase (phage lysin) (Trautwetter *et al.* 1986).

2.3. Lysis of the bacterial host by bacteriophages

Some phages with a small size like coliphages ϕX174 and MS2 (Markert & Zillig 1965, Lubitz *et al.* 1981, Bläsi *et al.* 1983, Atkins *et al.* 1979, Beremand & Blumenthal 1979) encode only one lysis protein; a membrane-spanning hole-forming protein (holin) that at the end of phage infection causes sudden collapse of the turgor pressure yielding to release of cell contents and mature phages leaving empty cells with relatively intact cell walls (ghosts) (Witte *et al.* 1990). Degradation products of the bacterial cell wall can be detected during the phage ϕX174 infection indicating that (localized) cell wall hydrolase activities of the host bacterium are involved in bacterial lysis (Lubitz & Plapp 1980). Bacterial autolysins may play an important role also on release of larger phages. For example, an autolysis deficient strain of *Streptococcus pneumoniae* is very poorly lysed by the phage Dp-1 (Ronda-Lain *et al*. 1977). Large phages possess a two-part lysis system consisting of holin and cell wall hydrolase (generally called endolysins or lysins) (Young 1992). All the phage encoded cell wall hydrolases so far described lack the secretion signal typical for sec-dependent transportation system (Young 1992, Young & Bläsi 1995). Therefore, unless damages in the cytoplasmic membrane occur, lysin activity accumulates in the cytoplasm without lysing the host cell. Accumulation of endolysin activity is irrelevant to the actual scheduling of lysis at least in the case of phages lambda, T4, and T7 (Young 1995, Wang *et al.* 1996). At the end of the infection, phage holin permeabilizes the cytoplasmic membrane allowing access of the phage lysin to its murein substrate.

The lysis genes of the *E. coli* phage λ represent a typical organization of lysis genes of bacteriophages. Three lysis genes have be determined: gene *S* encoding phage holin, gene *R* encoding phage lysin (transglycosylase) and gene *Rz*. The genes *S* and *R* are necessary for lysis. Function of the protein Rz is unclear; it is required for lysis only in a medium containing excess of divalent cations. The lysis activity of the phage λ is regulated at several levels. All late genes are transcribed from a single promoter P_R

(Blattner & Dahlberg 1972, Daniels *et al.* 1988). Upstream of the lysis genes, several domains capable of forming stem-loop secondary structures are recognized. Those include one rho-independent and one rho-dependent terminator. The late promoter works constitutively, but the transcription is terminated at the terminators before accessing the lysis genes. In the presence of the delayed-early gene product Q transcription can continue to the lysis gene's area (Daniels *et al.* 1988). However, the transcriptional control of the lysis genes may not be the main mechanism that determines the timing of host lysis. Bacterial lysis during phage infection involves complex phenomena like protein-membrane interactions, membrane energetics, and translational control of phage lysis genes. Also, interactions of the lysins with cell wall polymers and holins with the cytoplasmic membrane are involved in lysis of bacteria by their phages. In order to get an idea of those interactions, a brief presentation of the structural components of the bacterial cell wall structure is given.

2.3.1. The bacterial cell wall

The bacterial cell wall consists of several distinctive structures. In Gram-negative bacteria like *Escherichia coli*, three layers separating the cytoplasm are recognized; the cytoplasmic (inner) membrane, the cell wall, and the outer membrane. Together these components correspond to the cellular integrity of bacteria. Between the cytoplasmic membrane and the cell wall, the periplasmic space containing uncrosslinked murein and a variety of enzyme activities is recognized. In Gram-positive bacteria the outer membrane is missing, but the murein layer may in some species be covered by a layer formed by crystallized S-proteins. The S-protein layer is typical for lactobacilli colonializing the gastrointestinal tract.

2.3.1.1. The murein layer

The murein layer in the bacterial cell wall forms a rigid, shape-determining structure. It contains roughly equal amounts of polysaccharide and peptide and can be thus classified as peptidoglycan. Mureins are complex heteropolysaccharides. The repeating unit of this heteropolysaccharide has been termed muropeptide (Weidel & Pelzer 1964). The sugar components form linear chains of alternating units of *N*-acetylglucosamine and *N*-acetylmuramic acid linked ß 1->4. Attached to the carboxyl group of each muramic acid by an amide linkage is a short peptide, L-alanyl-D-isoglutamyl-L-mesodiaminopimelyl-D-alanine (Fig. 2A). This composition is shared by nearly all Gram-negative bacteria as well as by a few gram-positive rods (Schleifer & Kandler 1972). The average glycan strand of *E. coli* is about 30 muropeptides in length (Glauner & Schwarz 1983). A molecule of lipoprotein is attached about every 10th muropeptide (Braun & Rehn 1969). Adjacent strands are cross-linked to each other through the peptide side chains. The murein layer of Gram-negative bacteria is relatively thin and its structure is mainly two-dimensional. Because the cell wall has to adapt to the growth of the bacterium, enzymes

responsible for its hydrolysis and synthesis have to be present in the growing cell. The outer membrane (in Gram-negative bacteria) is presumed to be firmly anchored to the sacculus by the covalently linked lipoprotein molecules embedded in the outer membrane. The cytoplasmic membrane of growing cells appears to be attached to the outer membrane at a few hundred sites termed adhesion or fusion sites (Duckwitz-Peterlein *et al.* 1977).

In Gram-positive bacteria the sacculi consist of multilayered sheets of murein crosslinked to each other. The short peptide cross-linking the sugar components may differ from that of the *E. coli* murein. The linker peptide in the murein of lactobacilli and lactic streptococci contains D-aspartic acid residues (Ikawa 1964) (Fig 2A). Beside murein, which corresponds to 50 % of the cell wall, other polymers like teichoic acids, teichuronic acids and lipoteichoic acids (in some bacterial species replaced by lipoglycans) exist in cell wall of Gram-positive bacteria (Fig. 2B). Teichoic acids are acidic polysaccharides containing uronic acid residues (Baddiley 1972). The teichoic acids and other wall polysaccharides are covalently attached to the peptidoglycan. Teichoic acids represent 20 to 50 % of the dry weight of the wall. *Bacilli*, *Lactobacilli*, *Streptococci*, *Staphylococci*, *Micrococci* and *Streptomyces* are escpecially rich sources of teichoic acids (Ghuysen & Hakenbeck 1994). In *Streptococcus pneumoniae*, teichoic acids and lipoteichoic acids have identical chain structures, but in most Gram-positive bacteria are unrelated entities (Fischer *et al.* 1993). Lipoteichoic acids and lipoglycans are associated with the outer leaflet of the cytoplasmic membrane. A functional correspondence between the lipoteichoic acids of Gram-positive bacteria and the lipopolysaccharides in the outer membrane of Gram-negative bacteria has been suggested (Ghuysen & Hakenbeck 1994). The occurrence and structure of teichoic and lipoteichoic acids can be of taxonomic importance. Teichoic acids are important antigens and bacteriophage receptors (Heaton & Neuhaus 1992). Beside murein, neutral polysaccharides (composed of rhamnose, glucose, galactose, and sometimes of galactosamine) and anionic ribitol or glycerol teichoic acids are the major components of the lactobacillal walls. As polyanions (negatively charged large molecules), teichoic acids may maintain the appropriate ionic environment (especially divalent cation sequestration) and pH gradient for membrane synthetic activities, oxidative and fermentation processess (Card & Finn 1983, Koga *et al.* 1984). The low pH thus generated may also inhibit peptidoglycan hydrolases from degrading murein. Certain bacteria (like *Streptococcus pneumoniae*) possess the ability to respond to environmental conditions by altering the nature of their wall polymers. Those changes may directly affect the activity of the cell wall hydrolyzing enzymes. For example, substitution of the lipoteichoic acid component choline with ethanolamine results in inability of *Streptococcus pneumonia* cells to separate during cell division, and in resistance to lysis by bacterial or phage-encoded lysins (Tomasz 1984, García *et al.* 1983b, 1984).

The structure and composition of the *Lb. delbrueckii* cell wall polymers is not well known. For the related species *Lb. acidophilus* and *Lb. helveticus*, more detailed data is available. In the *Lb. acidophilus* cell wall, all of the *N*-acetylmuramic acid residues are peptide linked. Neutral and anionic polysaccharides are associated with the peptidoglycan. A relatively low extent of peptide cross-linking (2.3 crosslinked peptide units in the peptide moiety) is observed. The neutral polysaccharide is composed of equivalent amounts of glucose, rhamnose, and galactose. It is covalently linked to some

muramic acid residues of the peptidoglycan by means of phosphodiester bridges. The teichoic acid (anionic polysaccharide) component is a mixture of (ß or α) 1,6-linked polyglucose polymers with side chains of α-glycerol phosphate residues attached through phophodiester linkages. The anionic polysaccharides do not form an integral part of the polymer backbones (Coyette & Ghuysen 1970). In the course of the bacterial growth, neutral polysaccharides are partly replaced by anionic polysaccharides. The teichoic acids and lipoteichoic acids of *Lb. helveticus* are glycerol phosphate polymers partially substituted with α-D-glucosyl residues. The α-D-glucosyl substituents are primarily responsible for the serological specificity (Knox & Wicken 1970).

2.3.1.2. The cytoplasmic membrane

The cytoplasmic membrane of *E. coli* consists of a phospholipid bilayer, which provides the hydrophobic barrier needed to allow for different concentrations of small molecules and ions relative to the growth medium. It is permeable only for water and small hydrophobic molecules (Maloy *et al.* 1981). Many of the proteins attached to it are involved in bioenergetic and biosynthetic reactions or in transport of specific solutes. Some proteins are tightly bound (integral membrane proteins), some loosely bound (peripheral membrane proteins) and some interact only transiently with the membrane (Neidhardt *et al.* 1987). pH and salt homeostasis are largely controlled by the components attached to the membrane. Also replication of the bacterial genome and proteins synthesis are associated with the membrane. The cytoplasmic membrane contains all the apparatus required for energy transduction and oxidative phosphorylation (Bragg 1980, Ingledew & Poole 1984). Its composition and energy state affects the timing and efficiency of lysis by phage lysis proteins (Young & Bläsi 1995). Poisons like cyanide or azide that affect the membrane energy state induce lysis of *Bacillus subtilis* (Jolliffe & Doyle 1981, Kemper *et al.* 1993) suggesting an important role of the cytoplasmic membrane in regulation of the cell wall hydrolyzing autolytic activity. Membrane associations have been described for autolysins and phage lysins (Díaz *et al.* 1989, Henrich *et al.* 1995).

2.3.2. Phage lysins

Endolysin (or lysin) is a common name for cell wall murein hydrolyzing enzymes having different enzymatic activities and substrate specificities. Lysozyme-like enzymes (muramidases) hydrolyze *N*-acetylmuramyl-1,4-ß-*N*-acetylglucosamine bonds. Lytic transglycosylases act on the same bond as muramidases, but further catalyze the intra-molecular transfer of the *O*-muramyl residue to its own C-6 hydroxyl group. *N*-acetyl-muramyl-L-alanine amidases hydrolyze the bond between *N*-acetylmuramic acid and L-alanine. Peptidases hydrolyze the peptidoglycan stem or bridge peptides (Fig. 2A).

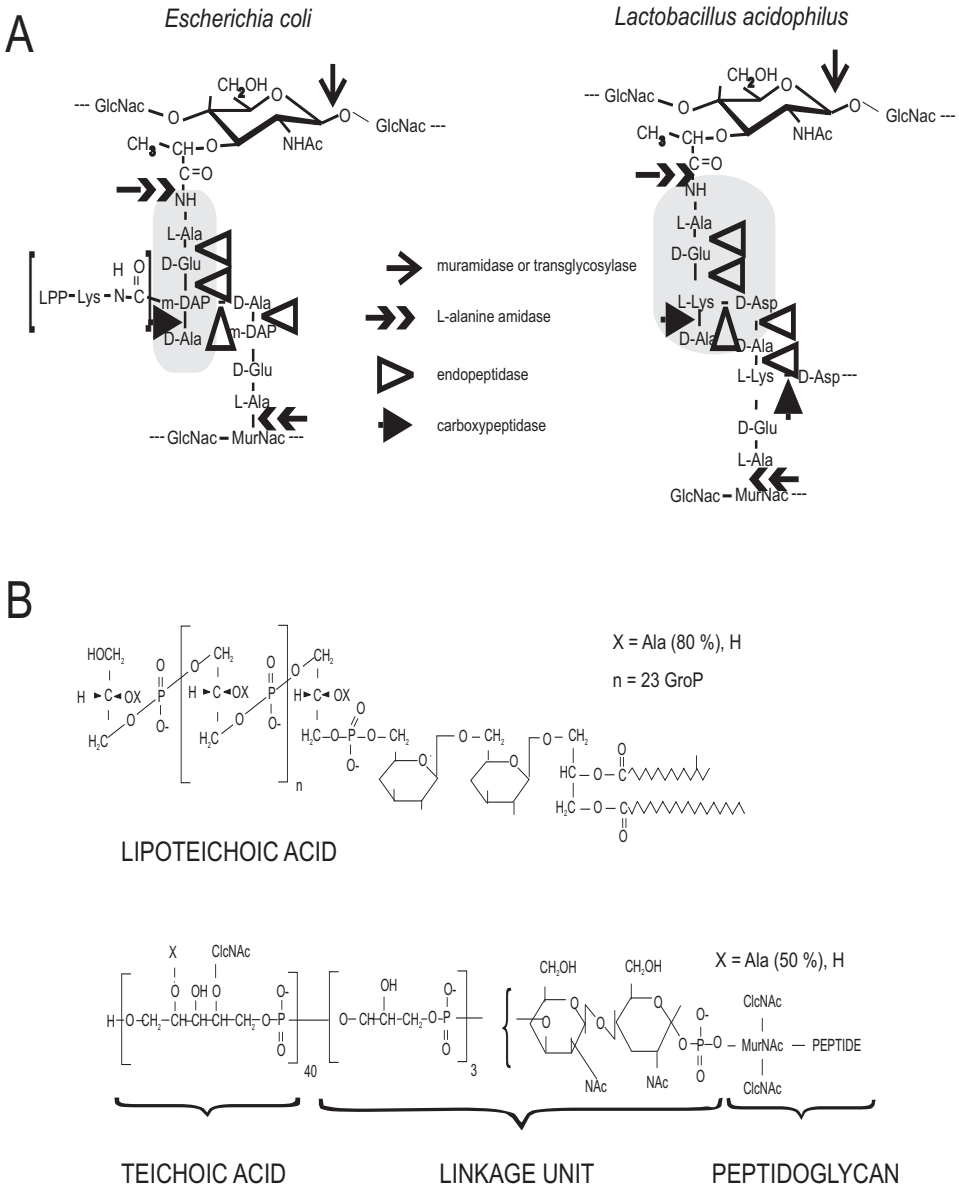

Fig. 2. A schematic drawing of the cell wall polymers. A) Muropeptides of *E. coli* (Gmeiner *et al.* 1982) and *L. acidophilus* (Coyette & Ghuysen 1970). The attack sites for different peptidoglycan degrading enzymes are shown. B) Lipoteichoic and teichoic acids of *Staphylococcus aureus* (Fischer 1994).

Phage lysins represent the same variety of enzymatic specificities as the host autolysins. Muramidase (e.g., coliphage T4 gene *e* lysozyme), transglycosylase (e.g., *R*-gene product of the coliphage λ), amidase (e.g., the gene *15* product of coliphage T7), aminopeptidase and endopeptidase (e.g., the lysin of the phage ϕ6 of *Pseudomonas phaseolicola*) activities have been detected (Tsugita & Inouye 1968, Bienkowska-Szewczyk & Taylor 1980, Inouye *et al.* 1973, Caldentey & Bamford 1992). A modular structure with separate domains for the murein degrading catalytic activity and for cell wall binding seems to be typical for lysins digesting the cell walls of Gram-positive bacteria. The lysis systems of phages infecting LAB are presently intensively studied (Tables 1 and 2) because of the potential biotechnological applications of the lysis genes and proteins. In the case of *Streptococcus pneumoniae*, the evolutionary relationship between the host autolysin LytA (*N*-acetylmuramoyl-L-alanine amidase) and phage lysins (muramidases) has been detected. For their activity, choline phosphate (component of the cell wall lipoteichoic acid) is needed. A repetitive amino acid sequence present in the carboxyterminal part of these enzymes is responsible for that substrate specificity (García *et al*. 1988).

2.3.3. Phage holins

Holins are membrane-spanning proteins that allow phage cell wall hydrolase (lysin) to access the cell wall murein by forming transmembrane holes. Defects in a phage holin gene result in either complete lack of lysis or inefficient lysis (Young & Bläsi 1995). Some small phages (e.g., the single-stranded DNA-phage ϕX174 and RNA-phage MS2) do not encode lysis proteins other than the holin protein (Markert & Zillig 1965, Atkins *et al.* 1979). Infection by these phages does not result in complete disruption of the host bacterium.The murein sacculus remains relatively intact, but loss of the cell content resulting in empty cells (ghosts) is observed. Holins are usually small and relatively charged proteins (Young & Bläsi 1995). In order to fulfill the membrane-spanning function, they contain at least one hydrophobic domain with a minimum length of 16 amino acids (Davis *et al.* 1994) to penetrate through the membrane lipid bilayer. Most holins can be drawn with two (or more) membrane-spanning domains separated by a potential turn region. In the case of the holin proteins S of the *E. coli* phage λ, a more ordered structure with up to three membrane-spanning domains has been proposed (Fig. 3, Raab *et al.* 1988). Some holins contain a highly charged N-terminus, which is a typical feature of holin-inhibitors. C-termini are either neutral or negatively charged. For λ and ϕX174 holins, there is experimental evidence that holins form multimeric structures during the hole formation (Zagotta & Wilson 1990, Bläsi *et al.* 1989). During normal infection, S protein accumulates in the cytoplasmic membrane throughout the late gene expression period. At the time of hole formation and lysis, app. $4x10^3$ S proteins are present in the bacterial membrane (C.-Y. Chang *et al.* 1995). Membrane vesicles prepared from S-containing membranes are permeable to charged molecules and incapable of active transport (Garret & Young 1982). There is no evidence for physical interaction or specificity between holins and lysins. Instead, lysins and holins from different origins may functionally replace each other. For example, the holin

Table 1. Characterized lysins of bacteriophages infecting lactic acid bacteria.

Phage	Lysin gene	Host	GenBank access	Enzymatic activity	Remark	Reference
Lactococcal phages						
ϕvML3		*Lc. lactis.*	X16178	muramidase	1,2	Oram & Reiter 1965, Shearman *et al.* 1989, 1991, 1994
c2	*gene 13*	*Lc. lactis*	L48605	muramidase	1	Mullan & Crawford 1985, Ward *et al.* 1993
Tuc2009	*lys*	*Lc. lactis*	L31364	muramidase	1	Arendt *et al.* 1994
ϕLC3	*lysB*	*Lc. lactis*	U04309	muramidase	1	Birkeland 1994
ϕUS3	*lytA*	*Lc. lactis*	M90423	amidase	1	Platteeuw & de Vos 1992
ϕbIL67	*gene13*	*Lc. lactis*	L33769	muramidase	1	Schouler *et al.* 1994
r1t	*lytR*	*Lc. lactis*	U38906,	muramidase	1	Nauta *et al.* 1996b,1997a
P001		*Lc. lactis*		muramidase	1,2	Hertwig *et al.* 1997
sk1	*ORF20*	*Lc. lactis*	AF011378	?		Chandry *et al.* 1997
Lactobacillal phages						
PL-1		*Lb. casei*	-	muramidase	2,3	Watanabe *et al.* 1984, Hayashida *et al.* 1987
ϕaADH	*lys*	*Lb. gasserii*	X78410	muramidase	1	Henrich *et al.* 1995
ϕg1e	*lys*	*Lb. plantarum*	X90511	muramidase	1	Kodaira *et al.* 1997
mv1	*lysA*	*Lb. delbrueckii*	M60167	muramidase	1	Boizet *et al.* 1990
Other LAB phages						
		Leuconostoc dextrenicum			2	Shin & Sato 1980

1) only sequence data, enzyme activity based on homology
2) enzyme purified
3) enzyme activity biochemically characterized

protein S^{21} of the *E. coli* phage P21, although unrelated to lambda S protein, can trigger lysis of *E. coli* expressing the phage λ lysin gene *R* (Bonovich & Young 1991). It is not known, whether the endolysin diffuses through those holes fully folded, or the folding and secretion of the lysin though the transmembrane holes occurs simultaneously. Surprisingly, several research reports of cloned holin genes don't demonstrate efficient cooperation of recombinant holin and lysin cloned into *E. coli* (Henrich *et al.* 1995, Oki *et al.* 1997). Because there are few small soluble basic proteins other than lysins free in the cytoplasm, Lu and Henning (1992) suggested that specificity of the holes to the lysins may depend on the sizes and net charges of lysins. Therefore Dabora and Cooney (1990) have suggested that by genetic engineering of the transmembrane holes forming proteins it might be possible to determine which proteins are released to the medium.

Holins of different phages are very divergent, and don't show much amino acid homology. All of them, however, contain at least one putative hydrophobic membrane-spanning domain that consists of amino acids that don't have charged side chains. When charged residues occur, they don't change the net charge of the transmembrane domain. The individual charges may be neutralized by intramolecular or intermolecular salt bridges (Young & Bläsi 1995). On the basis of the general structure, Young and Bläsi (1995) have categorized the holins of phages infecting Gram-negative bacteria into three types (Fig. 3). Type I holins (lambdoid holins) consist of at least 87 amino acids, and they have three or more putative transmembrane domains. The C-proximal transmembrane domain is not very hydrophobic. All lambdoid holin genes have a second Met codon at position three or four, and at least one of the two residues separating Mets is either Lys or Arg. Type II holins have the maximum size of 78 amino acids, and they have two potential transmembrane domains. In type II holins, both the N-terminus and the highly charged C-terminus may be disposed in the cytosol, while the two membrane-spanning domains may be embedded in the membrane lipid bilayer as antiparallel helices. The linker between the domains is either negatively charged or net neutral. T4 type holins are much larger, and seem to contain only one transmembrane domain. One transmembrane domain can be predicted for the protein e of phage ϕX174 with a holin function. At least seven apparently unrelated evolutionary lines of holin genes have been detected (Young & Bläsi 1995). In the case of phages of Gram-positive bacteria, at least four more unrelated holin families can be identified.

Also phages infecting Gram-positive bacteria exploit the two-part lysis system consisting of lysin and holin proteins. Some holins as, for example the *Bacillus subtilis* phage ϕ29, share several structural and functional similarities with the type I holins of Gram-negative bacteria (Young & Bläsi 1995). It has a dual start motif functional in *E. coli*, and up to three potential membrane-spanning domains. The holin of the *Lactobacillus gasserii* phage ADH holin is a type I holin of 114 amino acids (Fig.3B). It is interchangeable with the lambda S or ϕ29 holins (Henrich *et al.* 1995). Surprisingly, coexpression of ϕadh lysin and holin genes in *E. coli* does not result in lysis, suggesting that functionality of the lysin/holin system may depend on the expression host.

2.3.4. Scheduling of host lysis

Permeabilization of the cytoplasmic membrane by phage holin allows access of the cell wall hydrolyzing (phage lysin) activity to murein, which is followed by rapid lysis. Oligomerization of the holin monomers of phages ϕX174 (Bläsi *et al.* 1989) and λ (Zagotta & Wilson 1990) into the cytoplasmic membrane suggests formation of physical holes. Such data is not available for most holins. More generally, holins are thought to disturb the integrity and electric potential and thereby activate (possibly membrane-associated) phage lysins (Young & Bläsi 1995). Both holins and lysins may be produced by high amounts before the lysis occurs. Despite the extensive synthesis of the lysis proteins, late logarithmic and stationary phase cultures may fail to lyse, suggesting that the composition and energy state of the cytoplasmic membrane may affect lysis. In the phage λ, the holin protein S is constitutively synthesized after the onset of late gene expression that occurs when the delayed early protein Q begins to antiterminate at the $t_{R'}$ terminator immediately upstream of the 'lysis cassette' (Daniels *et al.* 1988, Chang *et al* 1993). There is no evidence for active regulation at the transcriptional level or at the level of the total translation of the holin gene during the vegetative cycle. Young & Bläsi (1995) have concluded that phages that employ a holin-endolysin system will employ anti-holin for the posttranslational regulation of holin function. In the case of lambdoid phage holins the inhibitor is encoded by the holin gene itself by the dual Met-start mechanism. The two S proteins (S107 and S105) have opposing functions. The shorter is the lethal holin while S107 acts as an inhibitor. Both forms are capable of membrane association (Bläsi *et al.* 1990). Temporal modulation of the ratio of the holin S105 and holin inhibitor S107 would conveniently explain how the lysis is scheduled. There is, however, no active regulation of the relative portion of translational starts at the dual Met start codons during the vegetative cycle (Chang *et al.* 1993). Hole-formation can be triggered by the addition of energy poisons, suggesting that the inhibitory capacity is dependent on the energized membrane. S107 may be more inhibited by the energized membrane (Steiner & Bläsi 1993). S107 inhibition of lysis works best when the total S protein levels are low (Bläsi *et al.* 1990). The overall charge of the amino acids preceding the membrane spanning domains may be important for timing of the lysis (Young & Bläsi 1995). The energized membrane regulates also the efficiency of phage ϕX174 holin. Bacteria grown in poor carbon source refuse to lyse by phage ϕX174. On the other hand, lysis can be accelerated by using energy poisons like azide. This suggests that the membrane insertion step requires highly energized membrane, while rapid decrease in the membrane energy level after the membrane insertion step of holin triggers the formation of transmembrane holes and lysis (Lubitz *et al.* 1981).

There is little experimental evidence on the presence of antiholin activity in most bacteriophages. The dual-start motif of genes encoding both holin and antiholin functions is characteristic of only a few (mainly type I and II) holins. The anti-holin protein may be encoded by a separate gene. In the coliphage T4, there is some evidence that the gene pair *t* (phage holin) and *r* (a potential antiholin) may fulfill this requirement. Lysis inhibition occurs only in cells in which transmembrane holes have not been formed (Bode 1967). The antitiholin protein r probably prevents the holin

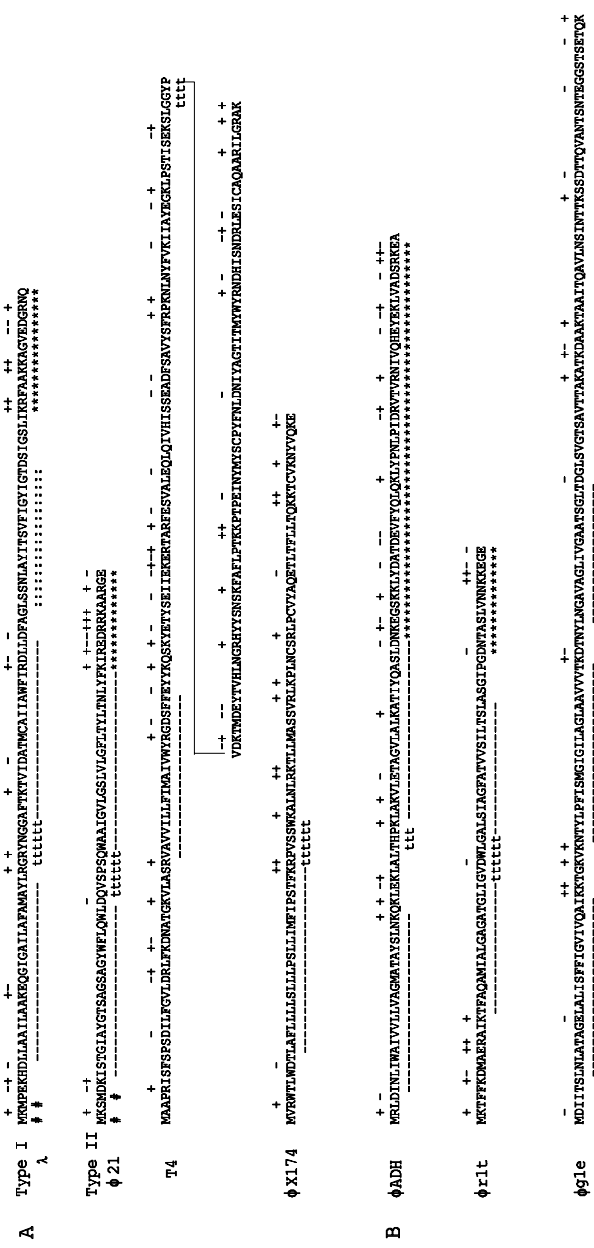

Fig. 3. Putative secondary structure of some phage holins. Holins of phages infecting (A) Gram-negative bacteria (B) Lactic acid bacteria. Membrane-spanning comains (--- and :::), helical turns (ttt), charged C-termini (***) and individual charges of amino acids (+ and -) are shown. Redrawn from Young & Bläsi 1995.

Table 2. Characterized holins of bacteriophages infecting lactic acid bacteria.

Phage	Host	Gene	Database access	Evidence	Reference
Lactococcal phages					
P001	*Lc. lactis*			experimental	Hertwig 1990, Hertwig *et al.* 1997
ϕUS3	*Lc. lactis*	ORF1	M90423	putative	Platteeuw & De Vos 1992
ϕvML3	*Lc. lactis*	within the lysin gene	X16178	experimental	Shearman *et al.* 1994
bIL67	*Lc. lactis*	ORF37	L33769	putative	Schouler *et al.* 1994
Tuc2009	*Lc. lactis*	S	L31364	putative	Arendt *et al.* 1994
ϕLC3					Birkeland 1994
c2	*Lc. lactis*	gene 12 or l17	L48605	putative	Ward *et al.* 1993, Jarvis *et al.* 1995
r1t	*Lc. lactis*	lytP	U38906	experimental	Nauta 1997, Nauta *et al.* 1996b
sk1	*Lc. lactis*	ORF19	AF011378	experimental	Chandry *et al.* 1997
Lactobacillal phages					
mv1, mv4	*Lb. delbrueckii*	lysB	Z26590	putative	Boizet *et al.* 1990, Dupont *et al.* unpublished
ϕadh	*Lb. gasserii*	hol	X78410	experimental	Henrich *et al.* 1995
ϕg1e	*Lb. plantarum*	hol	X90511	experimental	Oki *et al.* 1996, Oki *et al.* 1997

protein t from forming transmembrane holes, since lysis can be triggered with the energy poison cyanide (Josslin 1971). Bacterial genes affecting the membrane composition may also affect the holin function. Excessive free fatty acid accumulation in the cytoplasmic membrane of *E. coli* mutants may be incompatible with maintenance of the membrane potential and may thus trigger holin function (Bennet *et al.* 1971).

2.3.5. Evolutionary relationship between lysis proteins of phages and bacteria

Lysins of phages infecting different bacteria may share homology in the parts responsible for murein hydrolysis. For example, lactic acid bacteria phage encoded muramidases share homology with several fungal (*Chalaropsis*) type muramidase enzymes. Henrich *et al.* (1995) were able to detect four homology domains in the aligned sequences of five phage lysins. However, the carboxyterminal domains of lysins having different host species specificities were not aligned. Modular structure with separate domains for catalytic activity and substrate binding domains is typical for lysins of Gram-positive bacteria. Amino acid homology between the cell wall binding domains of the host bacterium's autolysin and phage lysins have been detected in *Streptococcus pneumoniae* (García *et al.* 1988, Díaz *et al.* 1990). These lysins consist of two functional domains; one responsible for the catalytic function (muramidase activity in phage lysins and amidase activity in the host autolysin) the other being responsible for the cell wall binding. The modular structure of bacteriophage lysins with separate catalytic and cell wall binding domains suggests a protein evolution mechanism, by which phages can rapidly accommodate to new host species (García *et al.* 1988). The genetic elements needed for integration of the phage genome to the host bacterium's chromosome (attachment sites) are located near the lysis genes in the genomes of many lactococcal and lactobacillal phages (Schouler 1996). That gene organization may increase the possibility that a temperate phage acquires its lysis genes from the host by improper excision of the phage genome from the host genome. The evolutionary relationship between host and phage lytic enzymes have not been observed in Gram-negative bacteria (Young 1992).

Phage holins are thought to represent the original phage lytic system, which has been enhanced during evolution by the acquirement of the murein hydrolase enzyme gene from the host bacterium. Amino acid homologies between holins of different phages rarely exist, although structural similarities can often be observed (Young & Bläsi 1995). Lau *et al.* 1987 have suggested a common evolutionary history for holins and colicins (membrane-spanning plasmid-encoded toxic proteins of some *E. coli* strains). For Gram-positive bacteria, no sequence data on the evolutionary relationship between phage holins and host proteins are available. Because the autolysins of most Gram-positive bacteria do not contain secreting signals, Young (1992) has suggested that holin-like proteins capable of transport of autolysins through the cytoplasmic membrane must be present in those bacteria. A holin type protein cid has been detected in *S. pneumoniae* by subjecting that bacterium to several rounds of penicillin selection (Moreillon & Tomasz 1988). Significant sequence similarities have been detected

between the holins of *Lc. lactis* phage ϕLC3, *Streptococcus pneumoniae* phage EJ-1 and *Staphylococcus aureus* phage ϕ11. This is the only example of sequence conservation in holins encoded by phages infecting unrelated host bacteria (Young & Bläsi 1995).

2.4. Purification of cell wall hydrolases

Beside hen egg lysozyme, only a few endolysins are manufactured on an industrial scale. These include the *Staphylococcus staphylolyticus* peptidase "lysostaphin" (Browder *et al.* 1965) and the *Streptomyces globisporus* muramidase M1 "mutanolysin" (Yokogawa *et al.* 1975, Siegel *et al.* 1981) that are delivered by the Sigma Chemical Co. Ltd. Cell wall murein hydrolyzing enzymes represent a variety of different enzyme activities and protein structures. Purification conditions and efficiency of individual purification systems may therefore greatly vary when applied to purification of different enzymes. Phage encoded lysins may be purified from the culture lysate. Bacterial autolysin activity can be purified from disrupted cells. However, in order to obtain reasonable production rates, both bacterial autolysins and phage lysins are usually cloned and overexpressed in a bacterial host. Phage lysins and many autolysins lack a signal sequence needed for translocation through the cytoplasmic membrane (Young 1992). Cell disruption is therefore needed for their purification. Mechanical cell disruption followed by solubilization of proteins by detergent and removal of the cell debris (for example by centrifugation or filtration) are normally the first steps of purification.

When processing large culture volumes (for example phage lysates), lysins can be concentrated and partly purified by precipitation with ammonium sulfate (Höltje & Thomasz 1976) or polyethylenglycol (García *et al.* 1983a). Also adsorption to the ion exchange resin in a stirred tank has been used (Tsugita & Iouye 1968, Bienkowska-Szewczyk & Taylor 1980). Chromatographic methods (usually preceded by removal of insoluble particles and dialysis) are commonly used for purification of lysins. Use of electrophoretic methods (like preparative polyacrylamide gel electrophoresis) is usually limited to preparation of small sample volumes for further analysis (e.g., amino acid sequencing). Ion-exchange chromatography is included in almost all purification protocols of lysins. In some cases, reasonable purity has been obtained by a single preparation step. For example, hen egg lysozyme can be separated and purified very selectively from egg-white by cation-exchange chromatography on carboxymethylcellulose with 92 % recovery (Rhodes *et al.* 1958). *Bacillus licheniformis* cell wall hydrolase cloned into *E. coli* could be purified to near homogeneity by ion-exchange chromatography using Toyopearl HW-55 superfine adsorbent (Kuroda *et al.* 1992). Most commonly, however, a sequence of different chromatographic methods like ion-exchange chromatography, gel filtration chromatography and affinity chromatography are required in order to achieve reasonable purity of the protein. Multiple purifications may significantly reduce the yield of the protein being purified. For example, partial purification of the *Bacillus subtilis* amidase was obtained by ion-exchange chromatography in hydroxyapatite followed by gel filtration chromatography on a Bio-Gel A-1.5 m column resulting in a 12 % yield (Herbold & Glaser 1975). Höltje

and Tomasz (1976) have purified pneumococcal amidase to biochemical homogeneity by using hydroxyapatite chromatography, Bio-Gel A-5m chromatography and preparative polyacrylamide gel electrophoresis resulting in a 3.4 % yield. Ursinus and Höltje (1994) purified membrane-bound lytic transglycosylase Mlt38 from *E. coli* by fast-protein liquid chromatography (FPLC) with SP Sepharose HP ion-exchange resin and by affinity chromatographies with heparin-sulfate and poly(U) as functional groups. This purification sequence was completed by MonoS ion-exchange FPLC and resulted in a 4.3 % recovery. Operation conditions and stability of the protein being purified influence the recovery of the enzyme activity during purification. An exceptionally high recovery (77 %) of fungus *Chalaropsis* lysozyme Ch *N*-acetylmuramidase was obtained by combination of ion exchange chromatography (Amberlite CG-50), gel filtration (Sephadex G-75) and crystallization in ammonium sulfate (Hash & Rothlauf 1969).

Purification of phage lysins is straightforward in those rare cases, where the enzyme is present in the mature phage capsid. For example, the lipid-containing *Pseudomonas* phage ϕ6 lysin (endopeptidase) can be purified from purified nucleocapsides by sucrose gradient centrifugation and ion exchange chromatography in a carboxymethyl-agarose column (Caldentey & Bamford 1992). Several phage lysins have been purified from phage lysates. Tsugita and Inouye (1968) purified the *E. coli* phage T4 lysozyme resulting in a 39.5 % yield by two ion-exchange chromatographies by IRC-50 resin followed by two purifications by gel filtration chromatography with Sephadex G-75. Bienkowska-Szewczyk and Taylor (1980) used a multistep purification system consisting of concentrating chromatography on Amberlite CG50I followed by two chromatographies on Amberlite CG50 I, one chromatography on CM Sephadex C-50 and gel filtration chromatography on Sephadex G-75. As a result, 12 % recovery of the enzyme was obtained. García *et al.* (1983a) purified phage Dp-1 lysin with 26 % recovery by PEG/NaCl precipitation followed by ultracentrifugation in CsCl gradient and gel filtration chromatography on Bio-Gel P-60. *Lb. casei* phage PL-1 encoded *N*-acetylmuramidase was purified by anion exchange chromatography (two passes on DEAE-Sephacel column) and gel filtration (two passes on Sephadex G-75 column) resulting in a 31 % yield. Cloning of phage lysins into an expression host like *E. coli* allows higher production rates compared to the use of phage lysates as the source material. *Bacillus subtilis* phage ϕ29 gene 15 encoded lysozyme produced in *E. coli* has been purified by CM BioGel A chromatography (Saedi *et al.* 1987). Partial purification (70 to over 90 % purity) of three *Bacillus cereus* phage lysins was achieved by gel filtration (Superdex 75) followed by anion-exchange chromatography on Mono-Q HR 5/5 (Loessner *et al.* 1997). Bacteriophage PRD1 encoded muramidase cloned into *E. coli* could be purified by fast protein liquid chromatography on a Mono S column (Caldentey *et al.* 1994).

Affinity chromatography is a specific and efficient purification method that has been successfully used for purification of some bacterial autolysins and phage lysins. It can be used either as the last purification step in a purification sequence or as an efficient single step purification method. In affinity purification of enzymes, the enzyme substrate is cross-linked to an inert purification resin. Choline crosslinked to Sepharose 6B adsorbent binds cell wall hydrolases that have an affinity with pneumococcal cholin-containing lipoteichoic acid, Forssman antigen (Briese & Hakenbeck 1985). It has been used for single step purification of streptococcal autolysin and phage lysins (García *et al.*

1987) and a chimeric lactococcal phage Tuc2009 lysin containing the cell wall binding domain of pneumococcal lysins (Sheehan *et al.* 1996). For most lysins of Gram-positive bacteria, the cell wall component recognized by the enzyme is not known. Therefore affinity purification can not be applied. However, fusion proteins capable of binding to substrates or cofactors of known proteins can be relatively easily constructed by using special expression vectors and mutagenesis methods. Substrate-binding amino acid sequences ("affinity tags") of enzymes like ß-galactosidase, maltose-binding or metal-binding proteins can be added to recombinant proteins. The location of the affinity tag has to be designed so that the biochemical properties of the fusion protein will not be disturbed. Therefore detailed knowledge of the structure of the recombinant protein is required. An affinity substrate cross-linked to a resin can be used for adsorption of fusion proteins (Guan *et al.* 1987, Sherwood 1991, Ford *et al.* 1991, Hochuli *et al.* 1987). Loessner *et al.* (1996) have constructed several biologically functional recombinant *Listeria* phage lysins that contain metal-binding polyhistidine sequence in their aminotermini. These added domains did not affect the biochemical properties of these enzymes, but allowed efficient purification of the gene product by metal chelate affinity chromatography with nicel-nitrilotriacetic acid (Ni-NTA) resin (Hochuli *et al.* 1987).

2.4.1. Expanded-bed adsorption chromatography

Ion exchange chromatography is one of the most powerful tehchniques for the purification of proteins in the biotechnology industry and is included in approximately 75 % of preparation schemes. An ion-exchange matrix consists of an insoluble matrix to which charged groups have been covalently bound. The charged groups are associated with mobile counter-ions. These counter-ions can be reversibly exchanged with other ions of the same charge without altering the matrix. Ion exchange chromatography has high resolving power, high binding capacity, and it is relatively cheap. Therefore it should be employed in the first steps of large scale purification processes (Bonnerjea *et al.* 1986, Chang & Chase 1995). In commercial downstream processing the protein to be purified is often found in a solution containing whole cells or cell debris. In both cases some form of clarification of the stream must take place before chromatographic techniques using packed bed chromatography can be employed (Chase 1994). Centrifugation and filtration methods, when performed on large scale, are expensive and labor intensive. Because the target protein may be present in a very low amount, several techniques capable of operating large volumes in the presence of suspended solids have been developed. In direct adsorption, through the use of the continuous stirred tank system, the capture of proteins may be inefficient because adsorption occurs in a single equilibrium stage process (Gordon *et al.* 1990, Chang *et al.* 1995). The use of fluidized bed adsorption can be used for efficient purification of bioproducts from unclarified fermentation broths (Bartels & Keliman 1958, Thömmes *et al.* 1995). However, the presence of mixing in either or both the liquid and adsorbent phases results in poor adsorption efficiency and low productivity. For efficient purification, it may be necessary to recycle the feedstream through the bed (Chang *et al.* 1995).

A very stable chromatographic bed can be obtained by using an adsorbent with a well-defined distribution of bead sizes and densities. In such beads, larger or heavier adsorbent beads will be located near the bottom of the bed, whereas the smaller or lighter adsorbent beads will be located near the top. This increases stability on the bed and the undesired circulation of adsorbent beads with back mixing of the liquid is avoided. A schematic comparison of an expanded-bed adsorption bed and a packed bed is shown in Fig. 4. Ordinary resins used in packed bed chromatographic purification are

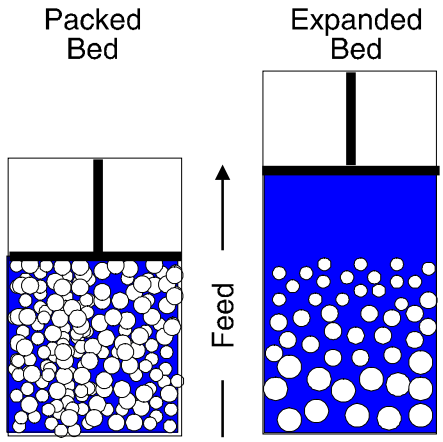

Fig. 4. Comparison between a) a packed bed and b) a stable, expanded bed. The diameter and size distribution of the adsorbent has been exaggerated for clarity. Redrawn from Chase (1994).

not particularly suitable for EBA. Adsorbents suited for use in expanded bed adsorption have a particle size and density large enough to allow 100 % bed expansion under conditions desired by industry (flow rates 200-300 cm/h). STREAMLINE adsorbents based on agarose containing quartz particles of a particle size range of 100-300 µm and density of approximately 1200 kg/m^3 have been introduced by Pharmacia Biotech (Uppsala, Sweden). The STREAMLINE matrix tolerates rigorous handling and can be re-used several times. Before new purification can take place the resin needs to be thoroughly cleaned, because viable cells and crude cell debris may be present. This "cleaning-in-place" is carried out by sequential irrigation of the resin bed in a column with solutions containing reagents such as 1 M NaOH, 2 M NaCl, distilled water at neutral pH, 70 % ethanol, 6 M urea, or 30 % isopropanol (Chase 1994). The STREAMLINE matrix can also be used as a start material for construction of affinity matrices (Chang & Draeger 1992). For example Chang *et al.* (1995) were able to perform immobilization of triazine dye into the STREAMLINE matrix, and purification of glucose 6-phosphate dehydrogenase with the resulting matrix. Because matrices used in EBA have to tolerate harsh treatment during the cleaning-in-place procedure, there is not yet a good selection of different affinity matrices available.

Some cell wall hydrolases are clearly cationic proteins (Rhodes *et al.* 1958, Noppe *et al.* 1996), and have a great potential for being purified by EBA using a cation-exchange resin STREAMLINE SP. For example, hen egg lysozyme has been used as a model protein for determination of properties (e.g., binding capacity) of ion-exchange resins and running conditions for ion exchange purification (Chang & Chase 1995). Expanded bed adsorption has also been successfully used for purification of equine milk lysozyme (Noppe *et al.* 1996).

2.5. Exploitation of phages in biotechnology

2.5.1. Phage regulatory elements in gene expression technology

Several cloning vectors are either derivatives of bacteriophages (e.g., phages λ and M13 of *E. coli*) or contain some elements of phage origin (e.g., cosmids and phagemid vectors). Site specific integration elements (attachment sites) in the genomes of temperate phages and host bacterium (*attP* and *attB*, respectively) can be used for stable insertion of cloned genes into the bacterial genome. Such integration vectors have been developed for a variety of organisms. Phages are a potential source of genetic elements for vector construction. During recent years integration elements of lactobacillal phages have been intensively studied (Raya *et al.* 1992, Fremaux *et al.* 1993, Dupont *et al.* 1995).The integration elements of phage mv4, a temperate phage closely related to the phage LL-H have been exploited for construction of a vector capable of integrating into the genome of *Lactobacillus plantarum* (Dupont *et al.* 1995). In the absence of a functional transformation system, this site-specific integration system has not yet been exploited for genetic manipulation of *Lb. delbrueckii*.

Efficient regulatory elements (e.g., promoters, operators, repressor protein encoding genes and terminators) of the phage genome have been exploited in gene expression technology. After completing the early genes' function (DNA replication, production of proteins needed for late genes' functions), efficient production of structural proteins and lysis proteins is launched. Promoters directing the expression of phage structural genes are used in gene expression technology. The strong promoters p_L and p_R of phage lambda (Blattner & Dahlberg 1972, Remaut *et al.* 1981, Elvin *et al.* 1990) and the promoter locating in front of the phage T7 major capsid protein encoding gene10 (Dunn & Studier 1983, Tabor & Richardson 1985, Mead *et al.* 1986, Studier *et al.* 1990) are widely used in *E. coli*. Phages may change the specificity of the host RNA polymerase towards phage promoters, or they produce their own RNA-polymerase that has affinity only towards phage promoters. The virulent coliphage T7 produces its own very effective RNA polymerase that has specificity only for the phage T7 promoters. When the gene encoding the phage T7 RNA-polymerase is expressed, transcription from the T7 promoters almost completely replaces transcription from native *E. coli* promoters (Rosenberg *et al.* 1987). Expression from T7 promoter has been utilized also in *Lactococcus lactis* (Wells *et al.* 1993). In temperate phages, expression of late genes (structural and lysis genes) is downregulated by repressor proteins, which by binding to

the operator sequence prevent transcription (negative regulation). Inactivation of repressor proteins leads to expression of the (cloned) genes downstream of the negatively regulated promoters. For induced control of the phage lambda promoter P_L in *E. coli* the temperature sensitive repressor protein mutant cI857 (Shatzman & Rosenberg 1988) is used. For lactococci, the repressor-operator system of phage r1t is well characterized (Nauta *et al.* 1996a, c), and thermosensitive mutants have been designed by molecular modeling of the lambda cI type repressor protein (Nauta *et al.* 1997b) That type of temperature-inducible regulatory systems hold promise for both fundamental and industrial applications that require controlled production of heterologous proteins in LAB.

Negative control of genes by a repressor-operator system is not typical for virulent phages. However, also in virulent phages like phage LL-H of *Lb. delbrueckii*, temporal changes in transcription of phage genes occur (early and late genes' expression). Switching on the late genes' function may occur through a mechanism in which transcription continues through terminator sequences that normally cause release of the RNA-polymerase from the template. For both temperate and virulent phages, antitermination mechanisms have been described (Roberts 1975, Daniels *et al.* 1988, Brody *et al.* 1983, Stitt *et al.* 1980). An antitermination system has been utilized for construction of a stable expression system (needed especially for cloning of genes expressing harmful proteins) for *E. coli*. In the system of Mertens *et al*. (1995) phage lambda derived terminator sequences upstream of the cloned genes prevent their expression. In order to switch on the cloned gene's expression, induced expression of the antiterminator protein (N) encoding gene of the pahage lambda is used. The antiterminator protein N has a short halflife (2 min), and a relatively high amount of this protein is needed for efficient antitermination and expression of the cloned genes. Also for phage LL-H, switching on the late gene's function may occur by expression of a phage-encoded antitermination protein, possibly encoded by the gene *ORF267* (Mikkonen *et al.* 1996b). Therefore, characterization of the promoter and terminator sequences of phage LL-H may benefit development of an inducible gene expression system for *Lb. delbrueckii*.

2.5.2. Applicability of lysis genes for lysis of bacteria

Recent advances in the purification technologies (like fluidisized beds and expanded bed adsorption methodology) permit the adsorption of proteins directly from the culture medium (Chase 1994). Such purification techniques are especially suitable for proteins secreted into culture medium. In order to make the intracellular or periplasmic located biomolecules accessible for purification, the bacterial cells must be disrupted. Cell disrupting methods suitable for small scale protein purification techniques (e.g., sonication, French press) may not be applicable for large scale industrial processes, where time, energy, and chemical consumption dictate the economy of purification. The drawbacks of the mechanical disruption include the need for cell harvesting, leakage of protease and peptidase activity that may degrade the proteins being purified to the medium during the sample treatment, and leakage of membrane-associated nucleic acids

resulting in high viscosity. The cell wall of (especially Gram-positive) bacteria may resist well mechanical disruption, and prolonged or multiple treatment passages may therefore be required.

Autolysis is not commonly used for disruption of *E. coli*, because its autolytic machinery is relatively inefficient. Autolysis in *E. coli* may be induced with osmotic shock, by inhibiting the cell wall synthesis, by aging, increasing temperature, or addition of organic compounds like butanol, ethyl acetate or toluene (Leduc & von Heijenoort 1980, Leduc *et al.* 1982, Dabora & Cooney 1990). During the osmotic shock treatment, extensive peptidoglycan degradation (65 to 70 % peptidoglycan degraded) can be observed. Some antibiotics (ß-lactams like penicillin) attach to the proteins involved in cell wall synthesis ("penicillin binding proteins", PBP). The rate of killing of *E. coli* by ß-lactam antibiotics is strictly proportional to the rate of bacterial growth. The highest rate of autolysis is seen in the exponential phase. Mutant strains that require added diaminopimelate or glucosamine (components of the cell wall) in their growth medium can be induced to lysis by deprivation of these components (Dabora & Cooney 1990). Under non-growth conditions (e.g., amino acid starvation), most autolysis inducers are not capable of triggering autolysis. The main difficulties in the methods based on triggering the autolysis are the need for exponentially growing cells and the low amount of cell wall hydrolyzing activity in *E. coli*. Efficient lysis may not be achieved with high cell densities. These methods may also require resuspending cultures in large volumes of aqueous solutions or the undesirable addition of antibiotics (Dabora & Cooney 1990). For small scale applications, the addition of cell wall hydrolyzing enzymes (e.g., hen egg lysozyme) can be used to remove the cell wall. This approach requires cell harvesting and usually permeabilization of the bacterial outer membrane (in the case of Gram-negative bacteria). Compared to added cell wall hydrolase preparates (like lysozyme), intracellular production of lysis proteins is therefore a better targeted alternative (Dabora & Cooney 1990). Two types of lytic proteins produced in bacterial cells can be used for lysis; cell wall hydrolases (lysins) and membrane-spanning proteins. As discussed early in the context of phage holins, the latter one may require the presence of some cell wall hydrolyzing activity in the bacterial cell. Membrane-spanning proteins applicable for bacterial lysis include peptic antibiotics produced by bacteria (e.g., colicins, lantibiotics) and phage holins. Colicins are antibiotic proteins produced in *E. coli*. Colicins E1, A, Ia, Ib and K form ionpermeable channels in the bacterial cytoplasmic membrane causing a decrease in active transport, loss of motility, reduction in ATP levels, and the arrest of protein and nucleic acid synthesis (Luria & Suit 1982). Several of these lysis genes have been cloned under controllable *lac* promoter. Induced expression of colicin genes to controlled lysis of *E. coli* is an attractive idea, but cloned colicin genes suffer from instability. For example, the gene *celB* encoding the colicin ColE2 is very lethal even in strains with the $lacI^q$ repressor background. To obtain a reasonable stability, the culture had to be cultivated at 30 ^oC (Pugsley & Schwarz 1983). Neither the *kil* gene from the *ColE1* operon could be maintained in plasmids if the bacteria were cultivated at temperatures greater than 30 ^oC. High toxity, poor clone stability, and inefficient cell wall hydrolysis obtained when killing bacteria with colicins reduce their applicability for lysis of *E. coli* cells (Dabora & Cooney 1990).

Cell wall hydrolyzing activity has been detected in most bacteria and yeasts. Some organisms like *Bacillus*, *Clostridia*, *Staphylococcus aureus*, and yeasts are readily autolysed. On industrial scale, autolysis has been used mainly for production of proteins, amino acids, and food flavouring components from yeast (Phaff 1977). Gram-positive bacteria, which possess an efficient autolysis mechanism, may be induced to lysis by "energy poisons" like arsein, cyanide, or azide. These substances activate the autolysis systems by disturbing the energy potential of the cytoplasmic membrane (Jolliffe & Doyle 1981). Autolysis may also be triggered by a change of culture conditions. For lactic acid bacteria used in cheese manufacturing, cooking and salting treatments are used to increase autolysis of the dairy starter bacteria in order to obtain a balance between lactic acid production (by viable bacteria) and peptidolytic activity by intracellular peptidases released from lysed cells (Crow *et al.* 1995a).

The first suggestion for the use of phage genes to obtain lysates containing intracellular enzymes was from Sher and Mallette (1952), who purified L-lysine decarboxylase and L-arginine decarboxylase from a phage lysate after infection of *E. coli* with phage T2. Auerbach and Rosenberg (1987) have patented the use of an *E. coli* strain containing defective temperature sensitive lambda lysogens as a method for cell disruption. The prophage lacks the genes for replication or structural protein assembly, and functional phages can not therefore be produced. The lysis genes are under temperature sensitive control through the use of the lambda p_L promoter and the cI857 repressor. The lysis could be induced in mid-log phase by a temperature shift to 42-44 ^oC. The phage lambda lysis genes *S* (holin), *R* (transglycosylase), and *Rz* cloned under control of the *lac* promoter cause rapid lysis within 40 minutes after induction of the gene expression with IPTG. In the absence of the functional holin gene *S*, lysis does not occur. *E. coli* can tolerate relatively high amount of intracellularly accumulated phage lysin (up to 2 % in the case of the phage T4 lysin) without lysis (Perry *et al.* 1985). Injuries in the cytoplasmic membrane by phage holin, freeze-thawing, osmotic shock or chemicals (like chloroform or toluene) yield rapid degradation of the cell wall and lysis of bacteria. Phage T7 lysin (amidase) has been used for construction of *E. coli* strains with increased susceptibility for lysis (Studier *et al.* 1990). Such strains can be conveniently used for externalization of intracellular gene products by osmotic shock treatment (Fidler & Dennis 1992). The T7 lysin has a dual function. Beside its cell wall hydrolyzing activity it downregulates phage T7 RNA polymerase (and thus expression of the genes cloned under control of T7 promoter) (Moffat & Studier 1987).

The efficiency of bacterial lysis triggered by phage ϕX174 holin depends on conditions like culture growth state, culture medium, pH, Mg^{2+} concentration, and the state or fluidity of the *E. coli* membranes. Lysis does not occur in stationary growth phase or in medium containing a poor carbon source (Markert & Zillig 1965). These conditions also affect the host autolysis, which suggests an important role of the host cell wall hydrolases (autolysins) for lysis. In small scale cell disruption it has been shown that function of the phage ϕX174 holin gene is more efficient than sonication at cell densities below $5x10^{10}$ cells/ml, but considerably less efficient at higher cell densities (Bläsi *et al.* 1984). Toxicity of the holin proteins and the high membrane energy requirement of their membrane insertion step have reduced the exploitation of the holin genes for lysis of *E. coli*.

2.5.3. Cell lysis of lactic acid bacteria and its contribution to ripening of the dairy products

Intracellular enzymes of the starter LAB are essential for complete conversion of the milk protein casein into the growth-promoting amino acids and the precursors for flavour. The enzymes essential for development of the flavour and texture typical for dairy products (especially cheese) include proteinases, peptidases, lipases, esterases and other enzymes. Especially amino acids, which are formed by aminopeptidases, have a great contribution for the flavour formation (el Soda 1993). Except proteinases that are associated to the cell wall or outer face of the cytoplasmic membrane, these enzymes have an intracellular location. The first steps of degradation of milk proteins are catalyzed by renneting enzymes (e.g., chymosin), milk proteases (e.g., plasmin) and by cell wall located proteases of the dairy lactic acid bacteria. During maturation of cheese these energy reserves of starter bacteria are quickly depleted (Visser 1993). Nutrients for growth of cheese non-starter (also known as secondary starters) lactic acid bacteria are provided by autolysed starter bacteria. Nonstarter lactic acid bacteria are essential for development of the final texture (e.g., the holes in Swiss cheese) and flavour in many cheese types. Lysis has influence on the growth rates and types of non-starter LAB (Crow *et al* .1995a). Modification of amino acids requires intact (viable) cells, and therefore it has been concluded that at that step, non-starter lactic acid bacteria may be more important than the starter bacteria. However, sufficient amounts of viable starter cells need to be present in cheese to affect the depletion of lactose and perhaps other important physiological reactions. The biocatalytic potential of starter bacteria for cheese ripening reactions could be modulated through autolysis of the starter cells (Crow *et al* 1995a).

A balance of autolysed and intact cells of lactic acid bacteria is important for the desired cheese ripening events (Fox *et al* .1996). Autolysis can be affected by sublethal heat treatment, sodium chloride concentration, salt-in-moisture values of cheese and by varying manufacturing condition, e.g., elevated cooking temperature (Lowrie *et al.* 1974). Proteolysis is the major factor in the ripening of many cheese types, influencing both texture and flavour. Hydrolysis of casein is obtained by milk proteases, the renneting enzyme and by the starter bacteria enzymes. Crow *et al* .(1995a) have concluded that the starter enzymes may be more stable in the cheese environment than in intact stressed cells. The cheese environment could be affecting stabilization as a result of higher substrate (peptide) concentrations or other factors such as salt concentration, pH, water activity. Intracellular peptidases released into cheese from starter lysis accelerate the peptidolytic steps and contribute to the higher levels ofamino acids in the cheese. Bitterness in cheese is a result of imbalance of the production of bitter peptides by protease and endopeptidase activities and their removal by exopeptidase activities. Bitterness control in cheese can be influenced by autolysis and its associated increase in peptidolysis (Crow *et al.* 1995a). Non-bitter cheeses are associated with increased starter autolysis and an increased concentration of amino acids. The simplified scheme of "how to make cheese fast" thus would involve a balance between lactic acid production and proteolytic degradation of milk proteins by addition

of exogenous enzymes. These kinds of trials have been made by the addition of "Neutrase" (*Bacillus subtilis* neutral protease) to the cheese. Substantial decrease in ripening time has been obtained (Fresta *et al.* 1995, Picon *et al.* 1995), but the flavour may not fully correspond to that of the original cheese brand. This indicates that a more sophisticated system using the components of the starter bacteria origin are required. The intracellular enzymes of the lactic acid bacteria are therefore an obvious source of the flavour producing activities.

The addition of purified cell wall hydrolase (hen egg yolk lysozyme) has been tested for lysis the starter strains. However, that approach is more efficient as a method to prevent the growth of contaminating species like *Clostridium* or *Listeria* than lysing lactic acid bacteria (Fox & Stepaniak 1993, Schneider *et al.* 1995). The cell wall of *Lb. delbrueckii* (and possibly of the majority of the lactic acid bacteria species) is relatively resistant to hen egg lysozyme. The modular structure of lysin with separate catalytic and cell wall binding domains implies the importance of cell wall binding for these enzymes. As the composition of the cell wall polymers (lipoteichoic and teichoic acids) that possibly have interactions with lysins are species (or genus) specific, a variety of effective enzymes may be needed for lysis of different lactic acid bacteria. Some alternative cell wall hydrolases are commercially available (like mutanolysin and lysostaphin), but their high prices do not allow their use as economical cheese ripening additives. The availability of low-cost cell wall hydrolases effective on the cell wall of LAB may facilitate controlled lysis of starter bacteria during cheese ripening.

The deliberate addition of phage to cheesemilk has been tested to lyse starter cells in cheese (Lowrie *et al.* 1974, Crow *et al.* 1995b). The extent of lysis could be regulated by using different amount of phages. No evidence for additional or alternative effects by phage on the ripening reactions was detected. Lysis by phage addition may be, however, mechanistically different from normal cell autolysis (Crow *et al.* 1995b). The lysin gene of the phage ϕvML3, when cloned into *Lactococcus lactis*, lyses the host when the stationary growth phase is obtained (Shearman *et al.* 1992). Shearman and Gasson (1992) suggested that considerable improvement of that system could be obtained if the expression of lysis genes was controlled by an inducible promoter. Indeed, presently a good variety of promoters inducible with pH, temperature or salt exist for *Lactococcus* (Israelsen *et al*. 1993, Sanders *et al*. 1995). One of the most promising inducible gene expression systems for lactococci is the nisin-inducible system (de Ruyter 1996). Nisin is a food-grade antibiotic produced by *Lactococcus lactis*, which when introduced in small, sublethal doses, can induce expression of the genes cloned under the control of the nisin-inducible promoter. Recently, de Ruyter *et al*. (1997) cloned the holin and lysin genes of phage ϕvML8 into *Lactococcus lactis* under control of a nisin inducible promoter. They were able to obtain nisin-inducible lysis of bacteria and acceleration of ripening of experimental cheeses. These very promising results wait for exploitation for larger scale cheese manufacturing. A similar scheme may be used for lactobacilli used for cheese manufacturing after the development of efficient cloning, expression and transformation systems. Several lactobacillal species as *Lb. salivarius* and *Lb. plantarum* produce bacteriocins, expression of which are, as is the case with nisin, induced by the bacteriocin itself (ten Brink & Holo 1996, Diep *et al.* 1996). Also the design of thermolabile repressor proteins of the lactococcal phage r1t (Nauta *et al.* 1997b) holds

the promise that similar inducible expression systems based on *Lactobacillus* phage material could be constructed.

2.5.4. Other putative applications for phage lysis genes and proteins

For genetic engineering, the methodology for delivery of (recombinant) DNA into the cell is a necessity. Beside carrying the foreign genes within phages (transduction), DNA can be introduced into cell by various methods, like protoplast transformation and electroporation. Only few bacterial species (e.g., pneumococci) can naturally intake foreign DNA. This competence may be artificially created for *E. coli* with treatment of cold calcium chloride (Mandel & Higa 1970). For most species, alternative means for genetic transformation are needed. Removal of the cell wall (an effective barrier preventing intake of large molecules) with lytic enzymes has been used to increase transformability of *Streptomyces*, *Bacillus* and *Streptococcus* (Bibb *et al.* 1978, Chang & Cohen 1979, Wirth & Clewell 1987). Watanabe *et al.* (1987, 1992) used purified phage PL1 lysin for preparation of protoplasts of *Lb. casei*, which could then be transformed with phage PL1 DNA. DNA can be introduced into protoplasts by the action of a fusant such as polyethylene glycol or by electroporation. The earliest successful result obtained by electrotransformation of *Bacillus cereus* (Shivarova *et al.* 1983) or plant cells (Fromm *et al.* 1985) were obtained by using protoplasts. The progress obtained by electrotransformation techniques (Chassy *et al.* 1988) has during the recent years reduced the need for the methodology of making protoplasts, because good transformability can often be obtained for intact cells. Whatever method is used, bacterial cells are usually most easily transformed during the logarithmic growth stage, when the autolytic enzymes are most active (Belliveau & Trevors 1989). This suggests that (localized) cell wall hydrolysis increases transformability. The availability of different cell wall hydrolases may promote genetic manipulation of poorly transformable species.

Genes for lysins that are active against pathogenic and spoilage bacteria may be used to engineer novel antimicrobial phenotypes in lactic acid bacteria. The lysin gene of a *Listeria monocytogenes* bacteriophage when expressed in *Lc. lactis* under control of the lactose regulated gene expression system resulted in production of antimicrobial activity lysing a wide range of *Listeria* strains (Payne *et al.* 1995).

Lysins active on cell walls of Gram-positive bacteria often have modular structures with a separate domain responsible for cell wall binding. Correlation between a bacterium's sensitivity to lysin and the structure of its cell wall polymers (e.g., teichoic acids, lipoteichoic acids, lipoglycans) may exist. Teichoic acids and lipoteichoic acids are important antigens (Sharpe *et al.* 1964). Therefore serological experiments and host specificity analyses of different cell wall hydrolases may give similar results. Cell wall hydrolyzing enzymes spotted onto a bacterial lawn (preferably bacteria cast in a soft agar layer) rapidly identify sensitive bacterial species on a plate (Boizet *et al.* 1990). Bacteriolytic activity has been successfully used for recognition of groups and species belonging to the genus *Enterococcus* (Berlutti *et al.* 1993).

Henrich and Plapp (1986) have suggested that a conditional lethal expression system based on phage holin could be used for detection of recombinant clones. Use of recombinant food-grade LAB as antigen-presenting vehicles has been extensively studied during recent years. Soluble antigens can be rendered more antigenic by presenting them to the immune system within inert particles or bacteria (Cox & Taubman 1984, Dahlgren *et al.* 1991, O´Haga 1994). Colonization by recombinant LAB will promote the immune response to an expressed antigen. Especially genetic constructs that lead to surface display are of considerable interest. Cell wall anchoring and membrane spanning domains of bacterial or phage origin may be utilized for those purposes. Leenhouts *et al.* (1996) have characterized the topological structure of the *Lc. lactis* phage r1t holin, which contains two membrane spanning domains. They were able to expose the antigenic epitope of the HIV-1 virus protein gp41 outside the bacterial cytoplasmic membrane by inserting it inbetween the transmembrane domains of the holin without loosing the functionality of the holin.

3. Outlines of the present study

Lb. delbrueckii, although an important species in the dairy industry, is not a genetically well studied organism. Plasmids that are commonly used as a raw material for construction of cloning vectors are rare in *Lb. delbrueckii*. The thick cell wall of this species is one major factor that makes its genetic transformation difficult. It also complicates purification of intracellular components, for example RNA, from this species. For these reasons, genetic engineering of *Lb. delbrueckii* has progressed slowly. The specific aims of this study were: (i) To study the possibilities of applications of phage LL-H properties for biotechnological use. In this respect, two genomic areas of LL-H of special interest, one encoding the phage LL-H major capsid protein and carrying a promoter of phage origin, and the other encoding the phage lysis genes were sequenced. (ii) To locate and characterize the genes and possible gene regulation elements in these DNA fragments. (iii) To characterize phage LL-H lysis genes, to study their expression and function in *E. coli*, and to analyse the encoded proteins. (iv) To develop a purification method for LL-H lysin protein. (v) To construct *E. coli* clones capable of stable maintanance and overexpression of the phage LL-H lysin gene. (vi) To test the applicability of the expanded-bed adsorption (EBA) method for a production-scale purification of phage LL-H lysin. (vii) To study how the phage LL-H lysis genes' (phage lysin and holin) expression could promote the disruption of *E. coli* cells and the release of intracellular proteins into the culture medium.

4. Materials and methods

Materials and methods are described in detail in the original articles (I-IV).

4.1.Bacterial strains, vectors and expression systems

Phage LL-H was isolated by the Valio Finnish co-operative Dairies' Association from a local dairy in 1972. Phage mv4 is a temperate phage present in *Lb. delbrueckii* subsp. *bulgaricus* lysogenic strain LT4 isolated in 1963. *Lb. delbrueckii* subsp. *lactis* LKT was used as the propagation strain for both LL-H and mv4. For sequencing of the phage LL-H DNA, phage DNA fragments were inserted into the M13mp18/19 vectors. As expression vectors, the plasmids pUC18/19, pTZ19, BluescriptKS/SK and pET21c were used. The *E. coli* strain JM101 (Yanish-Perron *et al.* 1985) was the host strain for M13, pUC, pTZ and Bluescript clones. Strains DH5α and K38 were hosts for pTZ clones. Strain MC1061[pT7POL23] (Casadaban & Cohen 1980, Mertens *et al.* 1995) was the host strain for the heat inducible expression system (Paper III), strains JM101 and BL21(DE3) the cloning hosts for lac-promoter inducible clones (Table 3). Induction of the cloned genes' expression was carried out by addition of isopropyl-ß-galactopyranoside (IPTG) (0.2 mM and 1 mM concentrations for the Bluescript and pET vector systems, respectively) or by shifting the culture temperature to 42 ^{o}C for 4 hours (in heat inducible clones).

4.2. Recombinant DNA techniques and DNA analysis

DNA cloning, ligation and transformation were performed according to standard procedures (Sambrook *et al.* 1989). The DNA-fragments for construction of efficiently phage LL-H lysin expressing clones were amplified by the polymerase chain reaction

Table 3. The clones used for identification of gene function and expression of lysis genes.

Clone	Cloning vector	*E. coli* host strain	Inducible promoter	Insert length / genes	Ref.
pUC18[2.5]	pUC18	JM101	*lac*	2.5 kb / *g34*	I
pTZlys	pTZ19	JM101	*lac*	3.1 kb / *hol*, *mur*	II
Bluescript[lacmur]	Bluescript-SK	JM101	*lac*	0.8 kb / RBS of hol, *mur*	III
Bluescript[T7holmur]	Bluescript-SK	MC1061 [pT7POL23]	*lac* and *T7*	1.3 kb / *hol*, *mur*	III
ET21[mur]	pET21c+	BL21(DE3) or BL21(DE3)pLysE	*T7*	0.8 kb / *mur*	III, IV

method (PCR) by using primers containing appropriate recognition sites for restriction enzymes used to linearize the expression vectors of *E. coli* (Papers III and IV). PCR was performed using *Taq*-polymerase (30 cycles of denaturation [1 min, 94 ^{O}C], annealing [1 min, 46 ^{O}C], and extension [2 min, 72 ^{O}C]) and 3 mM Mg^{2+} concentration.

Nucleotide sequences were determined in both strands by the method of Sanger *et al.* (1977). Phage LL-H DNA cloned into M13mp18/19 vectors was sequenced with Sequenase version 2.0 (United States Biochemical). The cycle sequencing technique modified from the method of Murray (1989) and TAQuence 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 using the Genetics Computer Group (GCG) Sequence Analysis Software Package Version 7.2-UNIX (Devereux *et al.* 1984), LKB DNASIS (V7.0) and LKB PROSIS (V6.02). Database searches were performed with the programs FASTA (Pearson & Lipman 1988) and BLAST (Altschul *et al.* 1990).

For detection and location of promoter(s) two methods were employed: S1 nuclease mapping and primer extension with AMV reverse transcriptase (Maniatis *et al*. 1982, Koivula *et al*. 1991).The phage LL-H RNA used for analysis was purified by the modified method of Slater (Slater 1984, Paper I).

4.3. Protein analysis and purification

For analysis of aminoterminal sequence of the phage proteins, proteins were separated in SDS-PAGE (Laemmli, 1970), electrotransferred onto a nitrocellulose membrane (type BA85, Shcleicher & Schuell) and stained with Ponceau-S as described in Paper I. The amino acid sequencing was performed on a modified Applied Biosystems 477A Sequencer (Baumann 1990). For analysis of the protein Gp34, it was necessary to perform digestion with trypsin, and isolate the peptides by reverse phase HPLC before

performing amino acid sequencing.

Cation exchange matrices CM-Sepharose C50 (for packed bed purification, Paper II) and Streamline SP (for purification by expanded bed chromatography, Paper III) of Pharmacia (Uppsala, Sweden) were used for chromatographic purification of the LL-H lysin protein. For packed bed purification, *E. coli* JM101 cells expressing the lysin gene *mur* were collected by centrifugation, resuspended in 20 mM sodium phosphate, pH 6.0, and sonicated. After removal of the cell debris, proteins were precipitated with ammonium sulphate, centrifuged, and the precipitate was dissolved in loading buffer (50 mM sodium phosphate, pH 6.5, 0.25 M sodium chloride). The sample was dialyzed against the same buffer overnight and applied to a CM-Sephadex C-50 cation exchange column. The column was washed with loading buffer, and proteins were eluted with a linear gradient of 0.2-0.8 M sodium chloride in 50 mM sodium phosphate, pH 6.5. Fractions containing lytic activity were pooled and concentrated by dialysis against dry polyethylene glycol 20,000. For preparing the sample for adsorption in expanded bed adsorption (EBA), sonication, chloroform-treatment supplemented with the murein-degrading activity of Mur itself, and coexpression of the gene together with the holin gene of the phage LL-H, and lysis triggering by thymol were tested (Papers III and IV, Table 5).

For determination of the enzymatic specificity of the LL-H lysin, samples containing 500 µg of purified cell wall material from *Lb. delbrueckii* and 25 µg of the purified LL-H lysin were incubated in lysis buffer (see paper II). The characterization of the reducing groups liberated by the enzyme was carried out essentially as described by Ward (Ward 1973). The samples were reduced with NaB^3H_4 (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, Bio-Rad, 1 x 20 cm). Samples containing either buffer, lysin or undigested cell walls served as controls.

5. Results and discussion

5.1. Sequence analysis of phage LL-H genes

In the present work, nucleotide sequence was determined and analyzed for two parts of phage LL-H genome: a 2497 bp region encoding the major capsid protein (Gp34) and a 2498 bp region encoding the phage lysis proteins (Papers I and II). These sequences have been deposited at GenBank under accession numbers L02496 and M96254. A summary of the characterized and putative genes is presented in the table 4.

5.1.1. Identification of phage LL-H structural proteins encoding genes

The nucleotide sequence of the 2497 bp DNA fragment (*Bam*H1-*Sal*1) capable of expressing the major capsid protein gene *g34* in the expression vector pUC18 (determined by immunoblotting with antiserum raised against purified LL-H particles) was obtained by the dideoxy sequencing method (Sanger *et al.* 1977) with the Sequenase enzyme and M13 as a cloning vector (Paper I). Five open reading frames were recognized in that fragment (Table 4). The largest of them was a 852 bp long *ORF* (designated as *ORF3* in Paper I) encoding a protein with predicted molecular mass of 31.3 kDa. The identity of that *ORF* as the main capsid protein encoding gene was confirmed by amino acid sequencing. Amino acid sequences of three peptides of trypsin digested protein Gp34 showed exact matches with the predicted amino acid sequence of the gene *ORF3* encoded protein. The aminoterminal methionine was missing from the mature protein indicating a posttranslational modification of the protein. Significant sequence homology with capsid proteins encoding genes of other phages (except the related *Lb. delbrueckii* phage mv4) was not detected.

The phage LL-H gene *ORF2* encoded protein with a predicted molecular weight of 19.6 kDa (Paper I) has significant homology (30 % identity and 56 % similarity in a 185 aa overlap) with the scaffolding protein encoding gene *11* of *Bacillus subtilis* phage SPP1 (Becker *et al.* 1997). Scaffolding proteins are required for assembly of the virus particles, but they are not present in mature phage particles. They may be, however,

observed in immature phage capsids (proheads). Phage lambda protein pNu3 (Georgopoulos *et al.* 1983) and phage T7 gene *9* encoded protein (Dunn & Studier 1983) are typical representatives of scaffolding proteins. Antiserum raised against purified phage LL-H particles did not detect a 19.6 kDa protein from purified phage particles or from the proteins encoded by the cloned 2.5 kb region (Alatossava 1987, Paper I) suggesting that the protein product of *ORF*-2 does not exist in a mature phage. However, antibodies raised against phage mv4 particles detected a 20 kDa protein from a corresponding clone expressing mv4 structural genes (Paper I). For preparation of antiserum, phage LL-H particles were purified in a cesium chloride gradient. Phage mv4 particles were purified from culture supernatant directly by ultracentrifugation, and may have therefore contained immature phage proheads (containing the scaffolding protein).The protein Gp20 immunoreactive with antiserum against phage mv4 is likely to be a scaffolding protein that does not exist in mature phage particles. On the basis of DNA or amino acid sequence comparison, no putative functions for the reading frames *ORF371*, *ORF144* or *ORF118* could be suggested (nomenclature after Mikkonen 1996; these genes are designated as ORFs 1, 4 and 5 in the Paper I) .

5.1.2. Identification and sequence analysis of the phage LL-H lysis genes

The lysis genes of phage LL-H were characterized by subcloning, functional analysis of the clones, and DNA sequencing. The presence of cell wall hydrolase (lysin) activity in the clones could be confirmed by spotting small samples of disrupted lysin-expressing *E. coli* cells onto a bacterial lawn (Boizet *et al.* 1990). In the case of clones expressing both the lysin and the holin genes of phage LL-H, lysin activity could be detected in the culture supernatant without disrupting the cells. The proteins of the phage LL-H lysate, of *E. coli* clones expressing phage LL-H lysin, and of appropriate controls were separated in a SDS-polyacrylamide gel containing *Lb. delbrueckii* cells under denaturing conditions (Leclerc & Asselin 1989, Paper II). After renaturation of the proteins in the gel, an app. 32 kDa protein capable of lysing the bacterial cells in the gel was observed in the *E. coli* clones expressing the LL-H lysin and in the phage LL-H lysate sample (Paper II). This band, which was not present in the *Lb. delbrueckii* sample, was concluded to be the phage LL-H lysin. Two additional cell wall hydrolyzing proteins (26 and 41 kDa) were detected in the phage lysate sample and *Lb. delbrueckii* sample. These proteins have similar sizes to the autolysins of *Lb. helveticus* (Sylvie Lortal, pers. comm.), and were concluded to be the autolysins of *Lb. delbrueckii* subsp. *lactis*. In the phage LL-H lysin expressing clones, an 894 bp long ORF was the only one capable of expressing a protein of the size suggested by SDS-PAGE analysis (Table 4). Amino acid sequencing of the purified protein Mur showed an exact match with the amino acid sequence deduced on the basis of the lysin gene's DNA sequence. The aminoterminal methionine was not present in the mature protein. LL-H lysin had significant homology with several phage muramidases, as judged by FASTA or BLAST search. 29 to 84 % aa identities in 178 to 297 bp overlaps were observed between the phage LL-H lysin and the aminoterminal sequences of muramidase of the following

phages: *Lb. delbrueckii* subsp. *bulgaricus* phages mv1 and mv4 (Boizet *et al.* 1990), *Lb. gasserii* phage adh (Henrich *et al.* 1995), *Streptococcus pneumoniae* phages Cp-1, Cp-7 and Cp-9 (García *et al.* 1988), and *Lactococcus lactis* phages LC3 (Birkeland 1994) and Tuc2009 (Arendt *et al.* 1994). 21 to 28 % aa identities in 178 to 209 aa overlap were found between the LL-H lysin and the muramidase type autolysins of fungus *Chalaropsis* (Fouche & Hash 1977), *Streptomyces globisporus* (Lichenstein *et al.* 1990) and *Clostridium acetobutylicum* ATCC824 (Croux & García 1991). These homological parts encode the murein hydrolyzing functions of the cell wall hydrolases. The carboxyterminal 87 amino acids of phage LL-H lysin did not contain homology with known cell wall hydrolyzing enzymes (except the very homologous lysin of phage mv4). Repetitive sequences characteristic for pneumococcal phage lysins are not present in phage LL-H lysin. Deletion of that part from the phage LL-H lysin decreased but did not completely destroy its cell wall hydrolyzing activity. However, the carboxyterminal parts of phage muramidases having different substrate specificities are different. Therefore it seems probable that these protein domains are involved in substrate (possibly cell wall lipoteichoic acid) recognition and binding. In pneumococcal phage lysins and lactococcal phage Tuc2009 lysin, the carboxyterminal domain binds to the choline-containing lipoteichoic acid of the bacterial cell wall (García *et al.* 1988, Sheehan *et al.* 1996). A lysine rich sequence KPKAEAKPKPKPK (amino acids 210 to 224) separates the putative N-terminal and C-terminal domains of phage LL-H lysin. As lysine-rich sequences are present in many membrane proteins (e.g., the sequence EPKPEPKPEPKPK is detected in the membrane protein M of *Streptococcus equi*, Timoney *et al.* unpublished), it is possible that this amino acid sequence facilitates membrane association of phage LL-H lysin. Association to the energized cytoplasmic membrane has been suggested to regulate the activity of bacterial cell wall hydrolyzing enzymes, and may also regulate activity of phage lysins (Kemper *et al.* 1993, Young & Bläsi 1995). Membrane association has been has been experimentally detected in the case of the *Lb. gasserii* phage adh lysin produced in *E. coli* (Henrich *et al.* 1995). A phage LL-H lysin expressing clone carrying a 3.1 kb DNA fragment leaked cell wall hydrolase activity into the medium suggesting the presence of a cytoplasmic membrane permeabilizing protein. The gene *ORF107* located upstream of (and partially overlapping) the lysin gene had a location typical for phage holins (Young 1992, Young & Bläsi 1995). It encodes a 107 aa long protein with an estimated isoelectric point (pI) of 11.1. Like holins, its encoded protein is rich in charged molecules and has a relatively small size (107 amino acids). It, however, does not contain dual Met-start codon typical for lambdoid holins. Like the holin of phage ϕX174, it has only one hydrophobic, putatively membrane-spanning domain (Fig. 5). In the holins of some other phages infecting lactic acid bacteria, e.g., the *Lb. gasserii* phage adh, *Lb. plantarum* phage g1e, *Lc. lactis* phages Tuc2009, LC3 and r1t, at least two putative transmembrane domains are recognized (Henrich *et al.* 1995, Oki *et al.* 1997, Arendt *et al.* 1994, Birkeland 1994, Nauta 1997). The structure of phage LL-H holin was different from the holin of the temperate *Lb. delbrueckii* phage mv4. Only the first 59 N-terminal

Table 4. Summary of the functions of the genes presented in this thesis. The nomenclature of the gene names is that used by Mikkonen (1996). The name used in the referred papers (Papers I and II) have been given in parenthesis.

Gene	Predicted mw of the protein product	GenBank Entry (nucleotides)	Function	Evidence	Reference
ORF371 (ORF1)	42.1 kDa	L29567 (#1591-2336) L02496 (#1-376)	unknown	-	Mikkonen & Alatossava 1994 Paper I
ORF178A (ORF2)	19.6 kDa	L02496 (#440-976)	scaffolding protein	sequence comparison, immunoblotting	Paper I Becker *et al*. 1997
g34	31.4 kDa	L02496 (#981-1838)	major capsid protein	immunoblotting, amino acid sequencing	Paper I
ORF144 (ORF4)	15.7 kDa	L02496 (#2030-2464)	unknown	-	Paper I
ORF118 (ORF5)	13.3 kDa	L02496 (#2461-2498)	unknown	-	Paper I Mikkonen & Alatossava 1994
ORF98 (ORFA)	10.3 kDa	L29568(#12910-12951) M96254(#1-261)	unknown	-	Paper I
ORF140 (ORFB)	15.5 kDa	M96254(#396-818)	unknown	-	Paper II
ORF112A (ORFC)	13.2 kDa	M96254(#808-1146)	unknown	-	Paper II
ORF107	11.6 kDa	M96254(#1133-1456)	holin	gene expression	Paper III
mur	32.6 kDa	M96254(#1446-2342)	phage lysin, muramidase	cloning, expression, purification, biochemical analysis	Paper II

amino acids of these holins shared homology. For these reasons, it was not initially regarded as a holin (Paper II). The function of the gene *ORF107* encoded protein as a phage holin was confirmed by subcloning and functional analysis (Paper III). Only the clones containing both the lysin gene *mur* and the gene *ORF107* leaked Mur-activity into the medium.

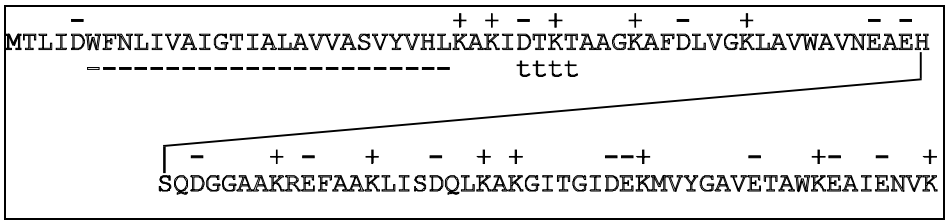

Fig. 5. The amino acid sequence and putative secondary structure of the phage LL-H holin. Charged amino acids (+ and -) and a putative helical turn (tttt) are shown. The hydrophobic sequence of 23 amino acids putatively forming the transmembrane domain has been underlined with a dashed line.

5.1.3. Sequence comparison between phage LL-H and mv4 genes

On the basis of Southern blotting analysis, approximately 60 % of the genomes of the phages LL-H and mv4 are at least 80 % homologous, the rest of the genome being totally different (Alatossava *et al.* 1995). The homology is located within the genome areas encoding late gene products; e.g., the structural proteins and lysis protein. Comparison of the DNA and amino acid sequences between the phages LL-H and mv4 showed that the structural genes and lysis genes and their encoded proteins are very homologous (Table 5). The major capsid protein and scaffolding protein encoding genes especially were very homologous (91 to 92 % DNA homology). DNA homology between the (putatively stuctural) gene *ORF144* and the corresponding phage mv4 gene was only 84 %, but most substitutions did not affect the protein structure (amino acid replaced by a functionally similar amino acid). The lysis genes were not as well conserved; 80 % DNA homology was observed between the lysin genes. Most of the nucleotide changes, however, either had no effect or only a slight effect on the amino acid structure (Table 5). Proteins encoded by the holin genes *ORF107* and *lysB* had different sizes (107 aa vs. 124 aa) and structures. They were homologous at their N-terminal parts (81 % identity in 59 aa overlap), but the carboxyterminal sequences were totally different. The putative transmembrane domains of these holins had similar locations and almost identical amino acid sequences (91 % amino acid identity). Further more, the charged amino acids residues were identical within the 59 aa long homologous region. In the case of LL-H, the holin gene *ORF107* overlapped with the lysin gene *mur* by ten nucleotides, but the corresponding genes *lysB* and *lysA* of the phage mv4 were separated by four nucleotides.

5.1.4. Determination of phage LL-H expression elements

The 3.4 kb phage LL-H fragment had been previously shown to be able to express the gene *g34* in *E. coli* irrespective to its orientation in the plasmid (Trautwetter *et al.* 1986). The presence of a promoter within the DNA sequence was therefore studied. By S1 nuclease mapping and primer extension method, transcription start point signals were discovered in front of the gene *g34* (primer extension results presented in Paper I). A putative -10 sequence (TTTAAT, six nucleotides upstream of the transcription start point) and -35 sequence (TTGACA) were detected. These sequences resemble the typical consensus sequences of *E. coli*, *Bacillus subtilis*, *Lactococcus lactis* (van de Guchte *et al.* 1991) and *Lb. delbrueckii* (Matern *et al.* 1994). That putative -35 sequence and the -10 sequence TTTAAT were spaced by only ten nucleotides instead of a typical spacing of 16-18 nt observed in the vegetative promoters of *E. coli* , *B. subtilis*, *Lactococcus lactis* or *Lb. delbrueckii* (Harley & Reynolds 1987, Moran *et al.* 1982, van de Guchte *et al.* 1991, Matern *et al.* 1994). Upstream of the promoter consensus sequences, a short inverted repeat sequence that might serve as a recognition element for regulatory DNA-binding proteins was found (Paper I). A weak putative transcription start signal (double underlined), a long A+T-rich sequence (bolded), and a hairpin structure resembling rho-independent terminators of *E. coli* (separated by << and >>, designated as T4 in Fig. 1) could be detected upstream of phage LL-H lysis genes (**AATAAATTATATTA**CTG<GAGCACC<TATGACG>GGTGCTC>TTTTTGATGAAAAAAGA AAGAAGAAAGAAA-holin). Long A+T rich sequences have also been detected upstream of lysis genes of the lactococcal phages r1t, Tuc2009, ϕLC3, and BK5T (van Sinderen *et al.* 1996, Arendt *et al.* 1994, Birkeland 1994). It is possible that expression of phage LL-H lysis genes is conrolled by an antitermination mechanism. One can not, however, exlude the possibility that some phage-encoded early protein binds to either DNA in front of the lysis genes or to the host bacterium's RNA polymerase making it capable of initiation of transcription of the lysis genes. Analysis of the transcription during phage LL-H infection suggests that the phage genome is transcripted in two sections. Because early genes are transcribed thoughout the infection cycle, Mikkonen (1996) suggested an antitermination mechanism as a control mechanism for expression of phage LL-H genes. Availability of cloning and research tools like promoter selection vectors for *Lb. delbrueckii* would enable the study of the functionality of promoter-like sequence in a proper genetic environment.

Table 5. Comparison of some conserved late genes between L. delbrueckii phages LL-H and mv4. When two names or numbers have been given, the first one refers to phage LL-H, the other to phage mv4. For amino acid sequence comparison, functionally similar amino acids were grouped by using the default values of the program PROSIS ver. 3.0 (Hitachi-Pharmacia).

Gene	function	size (nt)	DNA-homol. (%)	size of prot.(kDa)	pI	ident. aa (%)	homol. aa (%)
ORF178A/g20	capsid morphogenesis	534/540	92	19.6/20.1	5.7/5.9	85	88
g34	main capsid protein	852/861	91	31.2/31.3	6.0/4.8	88	92
ORF144	unknown	432/432	84	15.6/15.6	4.9/4.9	87	96
ORF107/lysB	holin	321/369	58 (78)*	11.5/13.6	7.7/11.1	50 (81)*	68 (98)*
mur/lysA	endolysin	894/891	80	32.6/32.6	10.1/10.1	85	90

* figures in parenthesis represent the values within the 59 aa aminoterminal homologous region.

5.2. Expressing phage LL-H lysis genes in *Escherichia coli*

The LL-H lysin protein Mur can be produced in *Escherichia coli* in relatively high amounts (up to 5 % of the total soluble intracellular proteins) without lysis of the cell. The induced expression of the gene *mur* did not retard the growth of actively growing cells. Because cell wall hydrolytic activity is needed for turnover of the murein (synthesis of new glycan strands and their insertion to the murein sacculus during cell growth and septation), increased concentration of Mur may be beneficial for actively dividing cells. However, *mur*-expressing cultures were unstable after reaching the stationary growth phase (results not shown). Clone stability could be enhanced by cultivating bacteria in minimal medium, that requires extensive biosynthetic activities (e.g., synthesis of amino acids) from the bacterium. Such conditions have been shown to also supress *E. coli* autolysis and phage-induced lysis (Markert & Zillig 1965).

Translational efficiency of the ribosome binding site of the lysin gene *mur* appeared to be poor in *E. coli*. The expression rate could be substantially improved by replacing the gene *mur*'s ribosome binding site with the ribosome binding site of the holin gene *ORF107*. After that replacement, up to 100-fold increase in *mur*-expression could be achieved by using the *lac*-promoter or *T7*-promoter based expression systems. Because the hairpin structure present upstream of the holin gene was not included in the modified sequence, increase in the expression rate is more likely be due to enhanced translational efficiency than increased stability of mRNA by a secondary structure in mRNA. Curiously, only slight improvement was obtained by the heat-inducible expression system of Mertens *et al*. (1995). It has been shown by Young *et al.* (1989) that heat treatment may decrease the efficiency of bacterial autolysis. The low amount of Mur-activity in the heat-inducible system was not due to formation of insoluble aggregates of Mur (inclusion bodies), as the amount of the protein Mur observed in a denaturing SDS-polyacrylamide gel was low (results not shown). It is not clear whether the poor yield of Mur-activity is due to a low synthesis rate or enhanced degradation of Mur during the heat treatment. Coexpression of the holin and lysin genes (and a consequent transport of Mur through the cytoplasmic membrane) in a heat-inducible system produced a much better Mur yield. Therefore it seems possible that intracellularly located Mur may be degraded by the heat-induced increased proteolytic activity present in the cell.

Expression of the phage LL-H holin gene did not cause observable physiological or morphological changes in *E. coli*. The bacterial growth was not affected by induction of the holin gene from the *lac*-promoter or from *T7*-promoter (results not shown). Its expression in an *E. coli* host strain containing lysogenized phage λ with a deficient holin gene *S* could not induce lysis (unpublished results). This suggested that the *ORF107* encoded protein can not fully functionally replace the phage lambda holin protein S. However, coexpression of the phage LL-H lysin gene *mur* and the gene *ORF107* yielded a decrease in culture turbidity, and filamentous bacterial aggregates could be detected in microscopy. Mur-activity, ß-galactosidase activity and isocitrate dehydrogenase activity could be detected in the medium (Paper III). These data suggested that the phage LL-H gene *ORF107* permeabilizes the bacterial cytoplasmic membrane for Mur. The possibility that high level of Mur could influence leakage of cell wall hydrolyzing activity into the medium was ruled out, because clones capable of

efficient expression of the phage LL-H lysin gene (but not containing the gene *ORF107*) without lysis could be constructed. It seems likely that both the expression and function of the phage LL-H lysin and holin are coupled. Addition of the nonionic detergent Triton X-100 is needed for solubilization of Mur suggesting a membrane association (Papers III and IV). Holin and lysin may attach to the cytoplasmic membrane immediately after their synthesis. Mur may have, like the *Lb. gasserii* phage adh lysin, a loose association to the cytoplasmic membrane that may stabilize the enzyme. Therefore it can be assumed that due to coexpression of lysin and holin genes, the local concentration of these proteins associated with the membrane yield efficient permeabilization of the cytoplasmic membrane and subsequent leakage of Mur to the periplasm. Dabora and Cooney (1990) have suggested that by genetic modification of holin proteins, both the timing of lysis and the size of the transmembrane holes could be affected. Inability of the phage LL-H holin to complete the defective holin protein (protein S) of phage lambda in *E. coli* could thus be due to an incompatible size of the transmembrane hole for the lysin. Although no experimental research supports the view that the holins have specificity for phage lysins, it is possible that the size and overall ionic properties of the lysin affects its ability to pass the transmembrane holes (Lu & Henning 1992). It is possible that membrane associations of these proteins occur immediately after protein synthesis. The adjacent or overlapping locations of the phage holin and lysin genes would ensure simultaneous protein synthesis, immediate association with the cytoplasmic membrane and high local concentrations of both proteins. All these facts may favour efficient lysis.

Cloned holin genes, like many other toxic membrane-spanning proteins encoding genes, may be unstable. Low stability of the clones was observed, when the genes *hol* and *mur* were cloned under control of *lac*-promoter (results not shown). The heat-inducible expression system of Mertens *et al*. (1995) maintained hol/mur-expression ability well. In the uninduced state, bacteria were grown at 26 ^{o}C. Low culture temperatures favour maintenance of toxic membrane-spanning proteins encoding genes. For example, Oki *et al.* (1996,1997) were able to maintain the *Lb. plantarum* phage g1e holin gene in *E. coli* at low cultivation temperature (25 ^{o}C). Temperature has been shown to affect the lipid composition (amount of saturated fatty acids), membrane fluidity and membrane energy (Cronan & Rock 1987). At low temperatures, the cytoplasmic membrane may be more resistant towards holins.

5.3. Induced cell lysis of *E. coli* by phage LL-H lysis genes

The phage LL-H lysin is not a secreting protein. It can not access the cell wall murein unless the cytoplasmic membrane is somehow damaged. During phage infection the permeabilization of the cytoplasmic membrane is achieved through the function of phage-encoded holin protein (Young 1992, Young & Bläsi 1995). As previously shown by Auerbach and Rosenberg (1987), phage lysis genes may be cloned into *E. coli* in order to obtain bacterial strains capable of lysis under certain conditions. The applicability of the phage LL-H lysis genes (the lysin gene *mur* and the holin gene *ORF107*) for lysis of *E. coli* was therefore studied. Coexpression of these genes could be

used for externalization of intracellular proteins of *E. coli* (Paper III). Actively growing and dividing cells seemed to be most readily lysed by phage LL-H holin and lysin genes. Host lysis was, however, inefficient in bacterial cultures having an optical density higher than 0.5 (measured at 600 nm, results not shown). Probably the efficiency of the phage holin function decreases with increasing culture cell densities. Bläsi *et al.* (1984) realized that expression of phage ϕX174 holin gene in *E. coli* results in efficient cell lysis only if the culture cell density was under 5×10^{10}. Bacteria grown in poor carbon source refuse to lyse by phage ϕX174. The membrane integration step of the membrane-spanning proteins (like colicins and phage holins) is probably dependent on the membrane energy. During late logarithmic or stationary growth of bacteria the energy state, the composition or the fluidity of the cytoplasmic membrane may not favor membrane integration of holins. Best externalization of the lysin protein Mur was achieved by performing coexpression of the genes *hol* and *mur* in a heat inducible gene expression system (results not shown). Heat shock is known to induce changes in composition and fluidity of bacterial membranes (Mejía *et al.* 1995), which may enhance the functionality of phage LL-H holin. Heat induction has, however, contradictory effects on bacterial lysis. Young *et al.* (1989) showed that in elevated temperatures lysis of *E. coli* by the phage ϕX174 occurs earlier, but on the other hand the autolytic activity of the host bacterium is substantially decreased. Judged by the poor intracellular accumulation of Mur in a heat inducible expression system, Mur may be stabilized by membrane association, which is disturbed by elevating the temperature of the culture. For large scale Mur-production or as a general lysis system for *E. coli*, better (i.e., culture density independent) methods were needed. In order to maximize the production of recombinant proteins, high culture densities are preferred in fermentation processes. Therefore, alternative methods for permeablization of the bacterial cytoplasmic membrane were studied. Small molecular weight substances that either destroy the membrane potential (energy poisons) or dissolve the membrane lipids could be used to release intracellularly accumulated cell wall hydrolases to their murein substrate. Chloroform-treatment disrupted *mur*-expressing bacteria very efficiently (Papers III and IV). Unfortunately chloroform is poorly soluble in aqueous media and has to be very thoroughly removed from the feedstock before purification of proteins in a chromatography column (Paper III). Rapid change in the membrane electric potential is thought to trigger lysis of the host bacterium during phage infection. Similar changes may be induced by "energy poisons" like cyanide, arsein, and azide compounds (Jolliffe & Doyle 1981, Young 1992). Potassium cyanide at 10 mM concentration did not lyse *E. coli* cells expressing the gene *mur*. Sodium azide treatment (75 mM) had to be supplemented with the detergent Triton X-100 in order to obtain lysis, indicating that the outer membrane of *E. coli* prevents the intake of azide into the cell. Alcohols are known to cause changes in the membrane electric state (Paterson *et al.* 1972). Thymol was found to functionally replace the effect of phage holin or chloroform-treatment in a cell-density-independent manner (Paper IV). The phage LL-H lysin Mur, although shown to be inefficient on the *E. coli* cell wall (Paper II), was able to lyse *E. coli* cells after the addition of chloroform or thymol. An increased amount of cell wall hydrolyzing activity inside the bacterial cells (by overexpression of the lysin gene *mur*) was necessary for lysis by thymol. Suprisingly, the *E. coli* strains BL21(DE3)pLysE and BL21(DE3)pLysS (Novagen) expressing a low amount of phage T7 lysin and supposed

to be easily lysed (Moffat & Studier 1987, Fidler & Dennis 1992), were not lysed by these treatments. Thymol was capable of penetrating the outer membrane and cell wall of *E. coli*. Being a bactericidal but nontoxic compound, thymol could be safely used for large scale lysis. Thymol was evenly dissolved in the medium (added as a stock solution in ethanol), and it did not disturb chromatographic purification of proteins (Paper IV).

The thymol-triggered lysis system described above could be well applicable for lysis of bacteria when purifying intracellular proteins produced in *E. coli*. The ability of thymol to trigger lysis without the presence of a membrane-spanning protein suggests that there is no need to clone the most harmful component of the phage lysis system, the holin gene. Bacterial clones containing phage holin gene may suffer from poor stability (Oki 1996, 1997). Instead of cloning all the phage lysis genes as is the case with the defective lambda lysogen (Auerbach & Rosenberg 1987), cloning of the lysin gene would be adaquate in a thymol-triggered lysis system. A lysin gene could be cloned under control of an inducible promoter and introduced into the bacterial cell by a plasmid or integration vector.

5.4. Purification of the protein Mur by cation exchange chromatography

By cation-exchange chromatography it was possible to selectively purify the recombinant protein Mur produced in *E. coli*. The weak cation-exchange resin carboxymethylcellulose (CM-Sephadex C-50, Pharmacia, Sweden) was used in packed bed chromatography and the strong cation-exchange resin STREAMLINE SP (Pharmacia, Sweden) in expanded bed chromatography (EBA). Only a few proteins of *E. coli* had an affinity with the cation-exchange resins at conditions ideal for adsorption of Mur (Papers II and III). Purification of Mur from a phage lysate (*Lb. delbrueckii* subsp. *lactis* infected by phage LL-H) by packed bed chromatography was not as successful due to several components of the culture medium (MRS lactobacilli broth, Difco) having affinity with the adsorption matrix. For purification of Mur in a packed bed column, bacterial cells were collected by centrifugation and then disrupted by sonication. The cell debris was removed by centrifugation, and the proteins were concentrated by ammonium sulfate precipitation and dialyzed against adsorption buffer (Paper II). The use of expanded-bed adsorption chromatography as a purification method substantially simplified the purification procedure, because clarification of the sample before loading into the purification column was not necessary. For EBA, the following sample treatment and purification variants were used: (i) Purification of Mur from sonicated bacteria, (ii) Purification of Mur from the culture medium after coexpression of the phage LL-H holin and lysin genes, (iii) Purification of Mur from the chloroform-treated culture by performing the adsorption step by a stirred-tank method, and (iv) Purification of Mur from the bacterial culture triggered to lysis by 5 mM thymol. Sonication efficiently disrupts bacteria but requires cell harvesting. Compared to the other cell disruption methods, sonication liberated higher amount of protein material that was not removed from the column during sample feed or washes (Paper III). Although the protein Mur is not particularly effective on the *E. coli* cell wall, it could be

used for disintegration of the host bacterium's cell wall (Paper III). Access of Mur to its murein substrate could best be achieved by chloroform or thymol treatment (Papers III and IV). Chloroform severely disturbed chromatographic purification by EBA by reducing bed expansion and producing turbulence (Paper III). Thymol-treatment efficiently triggered lysis of *mur*-expressing cells but did not interfere with bed expansion. A summary of the benefits and drawbacks of the purification variants is presented in Table 6.

Table 6. Effects of the bacterial disruption methods on chromatographic purification of intracellular proteins.

Cell lysis method	Benefits	Drawbacks	Putative applications
Sonication	Efficient lysis of any bacteria	Time-consuming, requires cell collection, not suitable for large sample volumes	Small scale purification
Chloroform-treatment	Efficient lysis	Chloroform disturbs bed expansion	Suitable for stirred tank adsorption
Coexpression of holin and lysin genes	No enzyme or chemical additive needed	Lysis efficient only for low culture densities	
Thymol-treatment	Lysis is not dependent on the bacterial growth state, does not interfere the chromatographic purication	-	Large scale lysis of bacteria

Phage LL-H lysin Mur is a cationic protein having a calculated isoelectric point of 10.4. Cation exchange chromatography has been successfully used for purification of cationic muramidases (lysozymes) from hen egg (Rhodes *et al.* 1958) and milk (Noppe *et al.* 1996). Lu and Henning (1992) claim that in a bacterial cell, there are very few small soluble cationic proteins free in the bacterial cytoplasm. Therefore, cation-exchange chromatography can be recommended as the first step when purifying (recombinant) endolysins with a cationic charge. Because ion exchange chromatography, by using the conventional packed bed system, is sensitive to any insoluble impurities, ion exchange chromatography can not always be used as the first purification method. EBA, however, is extremely suitable as the first applied purification method of proteins. By EBA, large culture volumes could be processed without removal of cell debris. Initial concentration of the sample (by precipitation with ammonium sulfate or polyethylenglycol, by ion exchange resin or ultrafiltration) used for purification of many phage lysins was not necessary (Höltje & Thomasz 1976, García *et al.* 1983a, Tsugita & Iouye 1968,

Bienkowska-Szewczyk & Taylor 1980). Compared to ion exchange in a packed bed, slightly better purification in a single step and higher yield (59 to 70 % compared to 46 %) could be achieved (Papers II and III). When performing ion exchange chromatography in a packed bed, it may be necessary to repeat chromatographic purifications in order to obtain a reasonable purity (Tsugita & Iouye 1968, Bienkowska-Szewczyk & Taylor 1980, Hayashida *et al.* 1987). In EBA, the adsorbent bed is stable and very little mixing of the adsorbent occurs during the purification. Therefore it has a better resolving power compared to chromatography in a packed bed (Chase 1994). As a method for purification of Mur, EBA chromatography appeared to be a nicely scaleable system. The full utilization of EBA, including good tolerance for cell debris and unbroken cells in the feed, could be achieved in a column designed for packed bed chromatography (Paper III). In that particular case, the adsorption step was performed by a stirred tank system. As a coarse and rapidly sedimentating resin, STREAMLINE adsorbent could be easily collected by decanting. In order to avoid blocking of the column, the sieve of the upper adapter was not installed. Adsorption in a stirred tank had the extra benefit that by collecting the adsorbent through decanting, most of the components possibly interfering with the chromatographic purification (as is the case for chloroform) could be removed. It can therefore be concluded that EBA can, with the modification discussed above, be used for purification of bacterial autolysins and phage lysins, either from the natural host system or from heterologous expression systems.

The good scaleability of EBA also means that the purification conditions described above may be easily adapted to an industrial scale. Methods like genetic transformation of protoplasts (Bibb *et al.* 1978, Chang & Cohen 1979, Wirth & Clewell 1987, Watanabe *et al.* 1987, 1992), release of intracellular components from bacteria (Loessner *et al.* 1994), or identification of bacterial species based on their sensitivity to purified lysin (Berlutti *et al.* 1993) will benefit from the commercial availability of new cell wall hydrolyzing enzymes like Mur. It should be noted that the relatively inexpensive and commercially available resin STREAMLINE SP was used for EBA. This resin tolerates well mechanical treatment and can be washed, regenerated and reused without needing to be removed from the chromatography column ("cleaning-in-place", see Chase 1994). The expanded bed system can be applied to affinity purification (Chase & Draeger 1992, Chang *et al.* 1995). For example, metal-chelate affinity chromatography of fusion proteins containing an added Ni-binding amino acid sequence (Hochuli *et al.* 1987) would be a powerful purification system if applied on a large scale. Such affinity matrices for EBA are not yet commercially available. Affinity purification methods can not be generally applied for purification of native autolysins or phage lysins.

5.5. Biochemical properties of the purified Mur

The purified phage LL-H lysin was biochemically determined as a *N*-acetylmuramidase (Paper II). The enzyme was specific on the cell wall of *Lb. delbrueckii* and its rather close relatives as *Lb. acidophilus*, *Lb. helveticus* (strain ATCC15009) and *Pediococcus damnosus* (Table 7). *E. coli* cell wall material used as a control in the biochemical analysis of the enzymatic activity of Mur was considerably resistant against Mur. It can

Table 7. Substrate specificity of the phage LL-H lysin protein Mur.

Species	Strain	Activity
Lb. delbrueckii subsp *lactis*	3 strains	+
Lb. delbrueckii subsp. *bulgaricus*	2 strains	+
Lb. acidophilus	ATCC 4356	+
Lb. helveticus	ATCC 15009	+
Lb. helveticus	ITG	(+)
Lb. casei	1/3 and 393	-
Lb. crispatus	ATCC 33820	-
Lb. gasseri	ATCC 33323	-
Lb. sharpeae	DSM 20505	-
Lb. curvatus		-
Lb. johnsonii	ATCC 333200	-
Pediococcus damnosus	E-86274	+
Propionibacterium freundenreichii	ATCC 6207	-
Lactococcus lactis subsp. diacetylactis	F7/2	-
Streptococcus pneumoniae	clinical isolate	-
Micrococcus luteus	clinical isolate	-
Bacillus subtilis	ATCC 27370	-
Clostidium sporogenes	ATCC 3584	-
Cl. tyrobutyricum	ATCC 25755	-
Escherichia coli	B, JM101	-

be reasoned that the composition of the cell wall affects the bacterial species sensibility towards Mur, and the cell wall composition among closely related bacterial species is likely to be similar. The pH optimum for Mur is between 5.0 and 5.5. pH values above 7 denature Mur irreversibly. Optimum temperature was around 30 ºC. The enzyme was however relatively tolerant against high temperatures; prolonged storage above 55 ºC was needed to inactivate it. Divalent cations Ca^{2+} and Mg^{2+} increased the activity of Mur. However, the amino acid sequence of Mur does not contain amino acid sequences characteristic for proteins binding divalent cations. Therefore it seems probable that divalent cations affect the interactions between Mur and the cell wall components. The cell wall teichoic acids bind the divalent cations, and competition of divalent cations

with Mur may prevent a too tight binding of Mur to the cell wall substrate. Association of lysins to the cell wall (lipo)teichoic acids is known to downregulate lysin activity (García *et al.* 1983b). Teichoic acids are, on the other hand, involved in cation sequestration. It is therefore possible that binding of divalent cations to the *Lb. delbrueckii* cell wall weakens the interactions between the cell wall components and Mur and that way increases its mobility and activity.

The sensitivity of those bacterial species to Mur is likely to reflect the similarities in cell wall composition, most probably the lipoteichoic acid structure. However, due to insufficient knowledge of the cell wall polymer composition in lactic acid bacteria, direct conclusion of the correspondence of the cell wall composition and sensitivity of the bacteria to the Mur can not be made. Some lactobacilli possess an S-layer on the surface of the cell wall and therefore express better resistance against lysis by Mur. From the two tested *Lb. helveticus* strains, the strain ITG that has a thick S-layer was more resistant to lysis by Mur. That layer is typical for species capable of adhering to the intestine.

The biochemical properties of Mur allow its use as a cheese ripening additive. Its pH optimum (pH 5.0 to 5.5) is near the pH in Emmental cheese. It is relatively thermoresistant allowing its addition to the cheese curd at any stage of cheese manufacturing (some cheese types are cooked at 50 to 60 ºC). The renneting enzyme chymosin did not decrease Mur-activity. If Mur is added to milk before the coagulation procedure, a majority (at least 90 %) of the Mur-activity is bound to the coagulated casein (results not shown). In the absence of an inducible lysis system based on cloned lysis genes, the addition of specific enzymes that accelerate the lysis of starter bacteria is a tempting idea. Fast and economical methods to produce and purify cell wall hydrolyzing enzymes (see Papers III and IV) active against dairy starter bacteria make that approach realistic.

6. Conclusions

In the present work, 4995 nucleotides (14 %) of the phage LL-H genomic sequence were presented. The function for the gene *g34* (major capsid protein encoding gene), gene *mur* encoding phage cell wall hydrolase (endolysin), gene *hol* (*ORF107*) encoding phage holin, the gene *ORF178A* encoding the capsid morphogenesis protein could be determined by biochemical or physical evidence. In addition, six ORFs with unknown functions were recognized. The promoter for the capsid protein encoding gene cluster was determined by primer extension and reverse transcriptase mapping methods. Due to the lack of efficient genetic engineering tools (like transformation methods and cloning vehicles), it is not yet possible to test the functionality of that promoter in *Lb. delbrueckii*. Therefore it is not clear, whether that promoter sequence could be used as an expression signal for construction of an expression vector for *Lb. delbrueckii*. There is so far no evidence of phage repressor mediated regulation for phage LL-H, which is a virulent phage. However, the presence of a putative antiterminator protein encoding gene *ORF267* (Mikkonen 1996), a rho-independent terminator-like structure immediately upstream of the phage LL-H lysis genes (Paper II), and a promoter for the capsid protein genes (Paper I) suggest that elements for construction of an inducible gene expression system are present in phage LL-H genome. Such a system could resemble that constructed by Mertens *et al.* (1995), in which expression of heterologous genes cloned downstream of a terminator sequence could be turned on by inducing expression of an antiterminator gene. Direct sequence comparisons between three structural and two lysis genes of the virulent *Lb. delbrueckii* phage LL-H and the temperate phage mv4 confirmed that, as suggested by Alatossava *et al.* 1995, these genes belong to the highly conserved genome areas. The proteins encoded by the structural genes *ORF178A*, *g34* and *ORF144* had 85 to 87 % amino acid identity. The holin proteins were more divergent (only 59 % aa identity). The putative transmembrane sequences of these proteins were almost identical suggesting their important role in a holin function. Also phage lysins were very homologous (85 % aa identity).

The 297 aa long phage LL-H protein Mur had homology with several characterized phage muramidases within its 210 aminoterminal amino acids (Paper II). No sequence similarities were observed in the carboxyterminal parts of phage lysins having different host specificities. This suggests a chimeric composition, in which the carboxyterminal parts of lysins may be involved in recognition of and binding to the host bacterium's

unique cell wall polymers. This kind of chimeric structure has been detected in pneumococcal autolysin and phage lysins (García *et al*. 1988). Lipoteichoic acids are known to regulate the activity of many bacterial autolysins and phage lysins (Tomasz 1984, García *et al*. 1983b, 1984) and may determine the substrate specificity of these enzymes. Detailed structure of the lipoteichoic acids of *Lb. delbrueckii* cell wall is unfortunately not known. The function of the carboxyterminal domain of Mur could be studied for example by construction of chimeric lysins having murein hydrolase and cell wall binding domains of different origins (Sheehan *et al*. 1996). In that way it may be possible to change or widen the substrate specificity of the cell wall hydrolyzing enzymes. The purified phage LL-H lysin was biochemically determined as a muramidase (Paper II). It was able to hydrolyze cell walls of *Lb. delbrueckii* and it's related species *Lb. helveticus*, *Lb. acidophilus* and *Pediococcus damnosus* (Table 7). The ability to remove their cell wall with a specific phage lysin protein holds promise for development of efficient transformation tools for these species, and may promote characterization of intracellular biomolecules and development of genetic engineering tools. The phage LL-H lysin gene *mur* could be easily maintained and overexpressed in *E. coli* indicating a potential for large scale production of the protein Mur in that host species. The two-part lysis system (holin+lysin) of phage LL-H was functional in *E. coli*. Expression of the phage LL-H holin gene (*ORF107*) alone or together with the phage λ transglycosylase gene *R* did not cause lysis of *E. coli*. Therefore it seems possible that well-integrated coexpression of phage LL-H lysin and holin genes (including almost simultaneous protein synthesis near the target site of the holin protein, namely cytoplasmic membrane) is required for efficient host lysis. In high culture densities, however, the holin function seemed to be poor.

Mur could be purified to near homogeneity by cation exchange chromatography (Papers III and III). For large scale purification of Mur, expanded-bed adsorption by the STREAMLINE system proved to be a fast, selective and well scaleable single-step purification method. The addition of thymol (an antiseptic substance that affects electric potential of membranes) could functionally replace chloroform or phage holin, and trigger bacterial lysis in a cell-density-independent manner (Paper IV). Chromatographic purification of Mur was not disturbed by thymol. Therefore thymol-triggered lysis of bacteria having an increased amount of either natural or recombinant cell wall hydrolase activity may probably be used as a general method for bacterial lysis for the purification of intracellular proteins.

Several biotechnological applications can be suggested for the purified Mur. It facilitates means for gentle cell lysis allowing extraction of easily degraded biomolecules, e.g., RNA from bacteria, and may thus promote the analysis of gene functions of certain lactobacilli (Mikkonen *et al*. 1996b). The bacterial cell wall barrier, which prevents intake of DNA, can be removed by Mur. Therefore Mur may allow the development of new transformation techniques. Species or group specific cell wall hydrolases may be used for recognition of bacterial species and for prevention of contaminating species (Berlutti *et al*. 1993, Scheider *et al*. 1995). Lysis of lactic acid bacteria is essential for the development of the texture and flavour of cheeses. Purified Mur may be used as an enzyme additive for accelerated cheese ripening. Alternatively, inducible lysis of *Lb. delbrueckii* may be obtained by cloning phage LL-H lysis genes

under inducible promoter. This approach has already been studied for *Lc. lactis* (de Ruyter *et al*. 1997).

Efficiency of different lysins on the cell wall of different bacteria may greatly vary. Unfortunately only few alternatives for hen egg lysozyme, the inexpensive and popular cell wall hydrolase, are presently available. These include mutanolysin (the muramidase of *Streptomyces globisporus*, Yokogawa *et al*. 1975, Kawata *et al*. 1983) and lysostaphin (the peptidase of *Staphylococcus staphylolyticus*, Browder *et al*. 1965). Phage LL-H encoded lysin presented in this thesis efficiently hydrolyses the cell wall of *Lb. delbrueckii*, an organism used for production of cheese and yoghurt. This enzyme, when produced and purified in a industrial scale, would widen the selection of cell wall hydrolases effective on cell walls of the economically important group of bacteria, lactic acid bacteria.

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