Aus dem Institut für Medizinische Mikrobiologie

Molecular investigations of peptidoglycan-binding proteins in *Listeria monocytogenes*

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Vorgelegt von Silke Machata
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Gutachter: Prof. Dr. Trinad Chakraborty

Gutachter: Prof. Dr. Alfred Pingoud

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Chatterjee, S.S., H. Hossain, S. Otten, C. Kuenne, K. Kuchmina, **S. Machata,** E. Domann, T. Chakraborty, T. Hain. 2006. Intracellular gene expression profile of *Listeria monocytogenes*. Infect Immun **74**(2):1323-38

Hain T., H. Hossain, S.S. Chatterjee, **S. Machata,** U. Volk, S. Wagner,. B. Brors, S. Haas, C.T. Kuenne, A. Billion, S. Otten, J. Pane-Farre, S. Engelmann, T. Chakraborty. 2008 Temporal transcriptomic analysis of the *Listeria monocytogenes* EGD-e sigmaB regulon. BMC Microbiol **28**;8:20

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Machata, S., T. Hain, M. Rohde, T. Chakraborty. (Poster) Simultaneous deficiency of both MurA and p60 proteins generates a rough phenotype in *Listeria monocytogenes*. DGHM, Göttingen, Deutschland (2005)

Machata, S., T. Hain, T. Chakraborty. (Poster) Characterization of mutants of putative murein hydrolases in *Listeria monocytogenes.* Meeting of the Network of Excellence (NoE) EuroPathoGenomics, Celakovic, Tschechien (2007)

List of Abbreviations

APS	Ammonium peroxydisulfate
ARP	Actin Related Protein
BHI	Brain heart infusion
BCIP	5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt
BSA	Bovine serum albumin
Bp	Base pair
°C	Degree celsius
Cfu	Colony forming unit
CRAMP	Cathelicidin related antimicrobial peptide
CSC	Cell surface complex
C-terminal	Carboxy-terminal
D-Ala	D-alanine
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide-5'-triphosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
FCS	Fetal calf serum
g	Relative centrifugal force
GAG	Glycosaminoglycan
h	Hour
IRAK	IL1 receptor associated kinase
IRF3	Interferon regulatory factor 3
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
kb	Kilobase
kDa	Kilodalton
L-Ala	L-alanine
LB	Luria-Bertani
LLO	Listeriolysin O
LGT	Prolipoprotein diacylglyceryl transferase
LPS	Lipopolysaccharide
LRR	Leucine Rich Repeat
LTA	Lipoteichoic Acid
M	Molar (mol/l)
MDP	Muramyl dipeptide
*m*Dpm	meso-diamino pimelic acid
min	Minutes
MOI	Multiplicity of infection
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
NEA	Non-essential amino acids
NF-κB	Nuclear factor-kappa B
NLR	NOD-like receptor
NOD	Nucleotide binding oligomerization domain
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PBP	Penicillin binding protein

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PG	Peptidoglycan
PRR	Pattern recognition receptor
rpm	Revolutions per minute
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulphate
TA	Teichoic acid
TAE	Tris/Acetate/EDTA
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline - Tween 20
TCA	Trichloroacetic acid
TE	Tris/EDTA
TEMED	Tetramethylethylenediamine
TIR	Translocated intimin factor
TLR	Toll-like receptor
Tris	Tris (hydroxymethyl) aminomethane
U	Unit
VASP	Vasodilator stimulated phosphoprotein
Xgal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

1. Introduction

1.1. Characteristics of *Listeria*

Listeria is a gram-positive, non-sporulating and facultative anaerobic rod, named in honor of Joseph Lister. It is commonly found in soil, stream water, sewage, plants, and food. The bacterium is well adjusted to its various habitats as it can grow at extreme pH and salt concentrations (31, 91). It also tolerates a wide temperature range (4 to 45°C), with an optimal growth between 30 to 37°C. *Listeria* has flagellar driven motility when grown at room temperature however flagellin expression is diminished at 37°C. It is catalase positive, oxidase negative and shows hemolytic activity on blood agar plates. The genus *Listeria* comprises two pathogenic species, *L. ivanovii* and *L. monocytogenes*, and the apathogenic species *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. grayi* and *L. murrayi*. It belongs to the firmicutes division, characterized by a low GC content (38%) and is closely related to *Bacillus subtilis* and staphylococci, and can be further distinguished via different serotypes, i.e. there are 13 serotypes known in *L. monocytogenes*. Serotypes can vary in expression of certain proteins and therefore show distinct phenotypes regarding hemolysis, virulence and growth. The serotype most frequently used for *L. monocytogenes* research is the sequenced wild-type strain EGDe serotype 1/2a.

1.2. Virulence of *Listeria monocytogenes*

Infection with *Listeria monocytogenes* occurs via ingestion of contaminated food and is often cleared rapidly before any symptoms are shown. However, *Listeria* is the causative agent of listeriosis, a serious disease with clinical manifestations such as septicemia, meningitis and abortion. The disease primarily affects immunocompromised adults, pregnant women and newborns and a severe course of infection has a mortality rate greater than 25 percent. Despite the ubiquity of *L. monocytogenes* in the environment the annual incidence of listeriosis (0.5-0.8 cases per 100,000 individuals) is very low (38). Whereas *L. monocytogenes* can cause listeriosis in animals and humans, *L. ivanovii* only causes the disease in animals, mainly sheep. The foods most frequently implicated as the cause of listeriosis include soft cheeses, dairy products, raw vegetables and cold fish or meat. The unusual growth and survival properties of *L. monocytogenes* are a serious concern for the food industry. Reports of listeriosis have increased since the 1960s, most likely due to an

extended cold-food chain, increasing lifespan and medical progress allowing immuno-deficient individuals to survive (131).

Following consumption of *Listeria*-contaminated food products, the bacteria enter the host through the gastrointestinal tract and penetrate the epithelial lining by crossing the mucosal layer to reach the bloodstream. From there *Listeria* infect the liver and spleen and at later stages they can spread to various organs and are able to pass the blood-brain barrier as well as the placental barrier.

Pathogenic *Listeria* are able to infect various cell types in the host. On the one hand they can spread among phagocytotic cell lines, such as dendritic cells (78) and macrophages (94). On the other hand they are also capable of infecting non-phagocytotic cells, such as epithelial cells, endothelial cells and hepatocytes (47-49, 167) by mediating cell entry.The means by which pathogenic bacteria mediate cell entry can be divided into two groups, the trigger and the zipper mechanism. The former is activated by bacterial proteins that are directly injected into the host cell via a type III secretion system. *Salmonella* and *Shigella* are examples of bacteria using this type of entry, mediated by Rho GTP-ases and Src signaling cascades. Induction of the zipper mechanism requires direct interaction between proteins exposed on the surface of bacteria and receptors on the surface of the host cell. This type of cell entry is used by *Yersinia* and *Listeria*.

Once inside the cell *Listeria* have to escape from the phagolysosome to be able replicate in the cellular cytosol. To this end, there is a set of virulence genes that enable the bacteria to enter, replicate and spread in the host cell. Several of these genes are located at the same chromosomal region forming a virulence cluster (Fig. 1) of 9 kb that is confined to the pathogenic species *L. monocytogenes* and *L. ivanovii*.

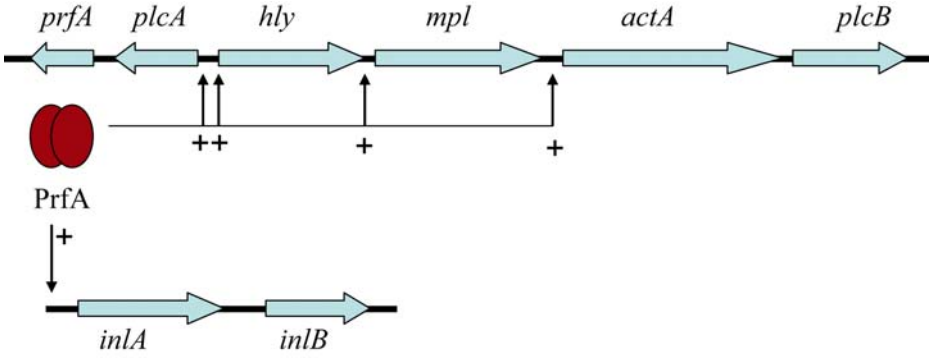


Figure 1: Virulence gene organization and the coordinate regulation by PrfA. Plus signs (+) indicate positive regulation of transcription by PrfA. (Adapted from reference (38)).

Among those genes are the transcriptional activator PrfA, two phospholipases (PlcA and PlcB), hemolysin (Hly), the surface protein ActA and a zinc-dependent metalloprotease (Mpl). These factors enable *L. monocytogenes* to persist and proliferate inside the cytosol of the host cell (27, 81, 127). Expression of the positive regulation factor PrfA, a protein of the Crp/Fnr-family of transcriptional activators, is controlled by an autoregulatory mechanism (26, 84, 87, 101). Activation of PrfA occurs by various environmental signals such as high temperature (37°C), stress conditions, contact with host cells and the eukaryotic cytoplasmic environment. PrfA not only controls the expression of the virulence cluster genes but also regulates a second cluster containing only two genes, *inlA* and *inlB*, that form an operon (Fig. 1).

The *hly* gene localized in the virulence gene cluster encodes listeriolysin O (LLO), a pore forming toxin protein with hemolytic activity (49, 160). A *L. monocytogenes* deletion strain Δ*hly* remains in the phagosome and is unable to escape attack of reactive radical derivates, mainly O_2 and NO^- by fusion with the lysosome (48, 83, 154). Therefore deletion of *hly* leads to attenuation of the bacterial strain. The hemolysin is crucial for the phagosomal escape of the bacteria into the cytosol of the host cell. LLO has a narrow pH range for optimal activity with its peak at pH 5.5 (125). Thus the hemolysin is well adapted to the intra-phagosomal environment, favoring lysis of the compartment. Once the phagosomal barrier is overcome the bacteria are released to the cytoplasm where the toxin is neutralized by the neutral pH in the cytosol. Another mechanism regulating the activity of hemolysin is the degradation of the cytosolic toxin that is mediated by host recognition of a PEST sequence in LLO (125). PEST (prolin, glutamic acid, serine and threonine) sequences are motifs recognized by protein degradation machineries for rapid destruction of the PEST-containing targets. It was shown that mutants of *L. monocytogenes* expressing LLO with absent PEST sequence secrete a toxin that accumulates in the cytoplasm of infected cells leading to cell disruption (35). The phospholipase A, encoded by *plcA*, acts synergistically with the hemolysin and supports the exit from the phagosome. Another listerial phospholipase C, encoded by *plcB*, enables *L. monocytogenes* together with LLO to pass through the double membrane surrounding the bacteria during entry of adjacent host cells. The metalloprotease, encoded by *mpl*, mediates maturation of a broad range phospholipase C (10).

Spreading of bacteria from one eukaryotic cell to another is achieved with the help of ActA, a bacterial surface protein that is encoded by *actA* in the virulence gene cluster. ActA copies the function of a eukaryotic family of actin nucleating factors, the Wiskott-Aldrich

syndrome protein (WASP) (125). These proteins bind monomeric globular actin and a seven-polypeptide complex comprising the actin-related proteins Arp2 and Arp3. The so-called Arp2/3 complex is the key player responsible for actin polymerization. ActA helps the bacteria to accumulate cellular F-actin by binding via the vasodilator-stimulated phospho-protein (VASP) with its proline rich repeat domain (25, 123) (Fig. 2). VASP is able to recruit the actin monomer by interaction with the protein profilin and modulates bacterial speed and directionality by controlling the geometry of the networks formed by the Arp2/3 complex (52). During cell division the F-actin is accumulated on the polar ends of the bacterial cell and can be polymerized to build an actin tail. Elongation of this actin tail after several cell division steps enables the bacteria to move inside the host cell and to spread to neighboring cells. How ActA distribution is linked to bacterial division and cell wall synthesis has been shown recently by Rafelksi and Theriot (128) by introducing their multistep model for passive polarization of ActA. First, ActA protein is secreted in several distinct spots along the cylindrical body of the bacterial cell away from sites of new cell wall synthesis. Next, ActA spreads over the cylindrical surface due to helical cell wall growth. Finally, ActA gradually accumulates at the hemispherical pole through slow incorporation of cylindrical wall material. The authors propose that the rate at which cylindrical cell wall material, and therefore also ActA, is incorporated into the poles is a function of the generational age of the poles. Poles of younger generations are thought to be more dynamic than those of older generations because of the dramatic change in growth rates as a rapidly growing septation zone gradually transforms into an inert pole over several bacterial divisions (128).

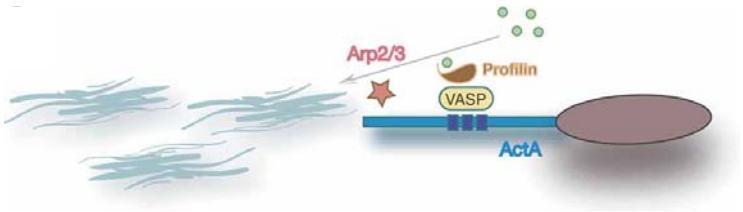


Figure 2: Polymerization of actin comet tails by ActA. VASP is bound by the proline-rich sequence of ActA and recruits profilin and actin monomers that will be polymerized by the Arp2/3 complex. The resulting formation of actin comet tails provides the intracellular mobility of *L. monocytogenes*. (Reprinted from reference (125) with permission).

The listerial proteins internalin A and B (InlA and InlB) are critical for cellular adhesion and internalization during infection of non-phagocytotic cell lines. Deletion of *inlA* and *inlB* abolishes the ability of bacteria to invade accordant cells. The internalins have

characteristic N-terminal leucine-rich repeat (LRR) domains (99) formed by tandem repeats of 20-22 amino acids that provide versatile binding motifs for protein-protein interactions. Recently, the existence of 25 members of the internalin family in *L. monocytogenes* has been reported (9). Proteins containing LLRs are divided into three families based on their association with the bacterial surface: LPXTG-internalins (i.e. InlA), GW or WχL-internalins (i.e. InlB) and secreted internalins (i.e. InlC). The characteristic C-terminal LPXTG motif covalently links proteins to the peptidoglycan (PG) of gram-positive bacteria and has been identified in 19 members in *L. monocytogenes*. The sorting signal of these proteins consists of the LPXTG sequence motif, followed by a hydrophobic domain of about 20 amino acids and a tail of positively charged residues. A membrane-bound transpeptidase, sortase A, cleaves the protein in the LPXTG motif between the threonine and glycine residues and links the carboxyl-group of the threonine to cell wall precursors. The second group of internalins comprises the two proteins, InlB and Lmo0549 (unknown function), that contain a C-terminal region responsible for non-covalent association to the cell surface. In the case of InlB, the motif is composed of three highly conserved modules of about 80 amino acids harboring the glycine-tryptophan dipeptide (GW modules). The C-terminal region of Lmo0549 does not contain GW modules but instead has a WχL domain associated with a recently discovered family of surface proteins, the Cell-surface-complex (Csc) family. Members of this family are thought to form a multicomponent complex at the bacterial surface and have been found in other low-GC content gram-positive bacteria such as *Lactobacillus plantarum* and *Enterococcus faecalis* (16, 144). Only two internalins, InlA and InlB, have been well characterized as crucial invasion factors for *L. monocytogenes* while four other internalins, InlC, InlH, InlJ and Lmo2026, have been reported to contribute in the infection process although not as invasins. Entry of *L. monocytogenes* into mammalian cells has been the focus of several recent reviews describing details of the interaction of InlA and InlB with the host (9, 11, 69, 125, 142).

The internalization pathway of InlA is illustrated in Figure 3A. InlA binds E-Cadherin, a calcium-dependent, transmembrane protein located on the surface of epithelial cells (i.e. Caco2) that plays an important role in cell adhesion, ensuring that cells within tissues are bound together. Interaction of InlA with E-Cadherin takes place in plasma membrane microdomains known as lipid rafts and leads to cytoskeleton rearrangement necessary for uptake of the bacterium into epithelial cells. Upon induction catenins are recruited to the bacterial internalization site: β-catenin binds to the C-terminal cytoplasmic

domain of E-Cadherin leading to recruitment of α-catenin, which is also dependent on a Rho-GTPase regulator, ARHGAP10. This protein interacts with the small GTP-binding protein Arf6. The α-catenin also mediates recruitment of the unconventional myosin VIIa and its ligand vezatin, generating the contractile force required for bacterial internalization. The human E-Cadherin receptor harbors a proline residue at position 16 that is required for InlA binding (85). Infection of mice by oral inoculation with *L. monocytogenes* has been proven unsuccessful due to the inability of bacteria to cross the intestinal epithelial barrier. Despite its similarity to human E-cadherin the mouse E-Cad receptor is inaccessible to InlA due to a substitution of glutamic acid for proline at position 16 (85).

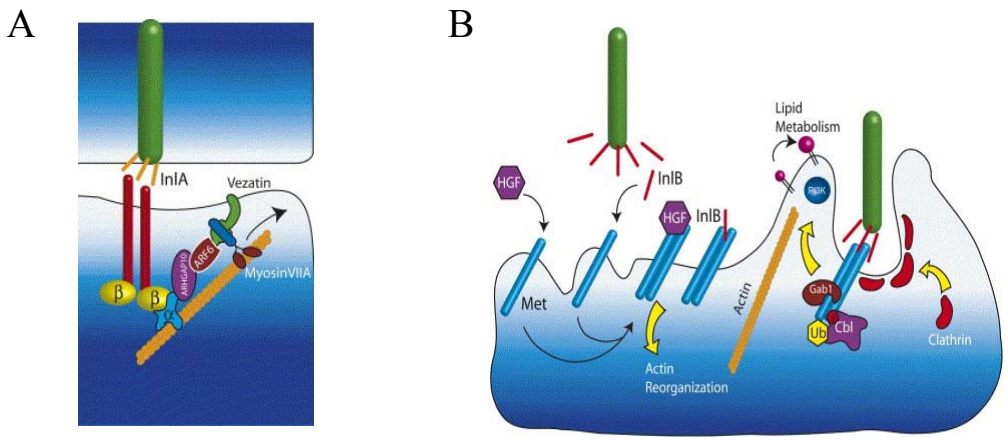


Figure 3: InlA and InlB signaling pathways in host cells. **(A)** InlA internalization pathway: The sortase-anchored protein InlA binds E-Cadherin and promotes the subversion of cell adherens junction machinery (including α- and β-catenins) to induce entry. The contractile force required for bacterial engulfment is thought to be provided by the myosin VIIA. The RhoGTase Rac1 contributes along with other molecules to actin polymerization. **(B)** InlB internalization pathway: The loosely cell-wall attached protein InlB interacts with receptors gC1qR and Met, which recruit several molecular adapters. These proteins induce the recruitment of PI3K and mediate ubiquitination of Met followed by clathrin-dependent endocytosis. (Reprinted from reference (11) with permission).

The internalization pathway of InlB is depicted in Figure 3B and described in the following. InlB promotes bacterial entry into a large variety of mammalian cells including epithelial cells, endothelial cells, hepatocytes and fibroblasts (161). Its anchorage to lipoteichoic acids (LTA) of the bacterial cell wall is non-covalent due to the C-terminal GW region. InlB triggers listerial entry by interacting with several ligands, e.g. glycosamino-glycans (GAGs), the ubiquitous gC1q receptor and, its major ligand, the hepatocyte growth

factor receptor (Met/HGF-R). Met is a receptor tyrosine kinase and plays a crucial role in organ morphogenesis, cell proliferation, cell migration and differentiation. The InlB-Met interaction activates signaling pathways that result in cytoskeletal remodeling of F-actin needed for bacterial uptake. InlB induces the tyrosine autophosphorylation of Met and the recruitment of several effectors as illustrated in Figure 3B. The recruitment of the adaptor proteins Cbl and Gab1 is induced, which in turn can recruit the p85/p110 class I PI 3-kinase (PI3K). Activation of PI3K leads to changes in the lipid composition of membranes and to formation of phosphatidylinositol 3,4,5, trisphosphate (PIP3), which initiates the re-organization of the actin cytoskeleton. Recruitment of the upiquitin-ligase Cbl induces ubiquitination of Met, mediating endocytosis of the receptor and of bacteria associated with it via clathrin-coated pits. Unlike InlA, InlB is able to bind multiple host components besides its primary receptor Met. InlB can be released from the bacterial cell wall upon contact with the glycosaminoglycan heparin of the extracellular matrix, followed by binding between heparin and InlB through its GW domains (73). This leads to enhancement of bacterial entry, suggesting a direct role for the GW domains in invasion. Soluble InlB can also interact through its GW motifs with a host-cell membrane molecule, the receptor for the globular head of complement C1q molecule (gC1q-R) (14). However the functional significance of this interaction is not yet understood.

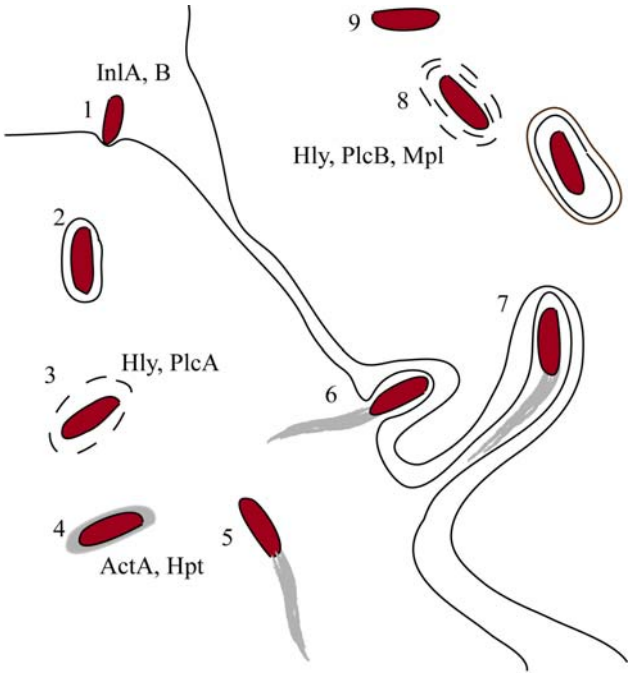


Figure 4: The infection cycle of *L. monocytogenes* in eukaryotic host cells and bacterial factors involved. The various steps during infection are summarized as follows:
1 Attachment
2 Internalization
3 Escape from endosome,
4 Proliferation and actin filament assembly
5 Tail formation and motility
6 Pseudopod formation
7 Pseudopodal uptake
8 Escape from double-membrane vacuole
9 Proliferation
(Adapted from reference (154)).

Another gene that has been found to be regulated by PrfA is *hpt* (*uhpT*) encoding a hexose phosphate transporter. The translocase Hpt is required for cytosolic proliferation by using hexose phosphates from the host cell as a source of carbon and energy (30). The infection cycle from cell entry to replication in adjacent cells is outlined in the diagram (Fig. 4).

As described above *L. monocytogenes* uses a series of proteins, which are either secreted or cell-wall attached, to interact with the eukaryotic cell and to mediate changes in the host system allowing entry and proliferation without being killed. Several recent studies have shown the role of surface proteins for virulence that are not under the control of PrfA, among those cell-wall hydrolases and lipoproteins (8). A prerequisite for better understanding the contribution of surface proteins for *L. monocytogenes* virulence is a better understanding of the biochemical characteristics of the bacterial envelope. The cell wall-associated modules in *Listeria* have therefore received further attention in this study.

1.3. Properties and structure of the bacterial cell wall

The cell wall of gram-positive bacteria has various functions, among those mechanical and osmotic protection. It also serves as a docking site for bacterial proteins that can interact with the environment and thus enables the cell to react to certain signals. Moreover, it is responsible, in part, for the permeability of proteins, the presentation of peptidoglycan hydrolases and adhesins and for cell surface hydrophobicity (109). The cytoskeleton of the bacterial cell wall consists of peptidoglycan (PG) or murein linked to various molecules such as teichoic acids, lipoteichoic acids, polyphosphates and carbons (Fig. 5B). The basic structure of murein is similar in gram-positive and gram-negative bacteria but the thickness of the cell wall differs: the gram-positive wall is at least 10 to 20 layers thick, whereas the gram-negative wall is composed of only 1 to 3 layers. PG is a polymer consisting of sugars and amino acids. The sugar component consists of alternating residues of the disaccharide β-(1,4) linked N-acetyl-D-glucosamine (NAG) and N-acetylmuramic acid (NAM) cross-linked by peptidic bridges (Fig. 5A). The peptide chain is cross-linked to the peptide chain of another strand of a neighboring glycan strand forming a net-like polymeric structure. In *L. monocytogenes* this cross-link is directly between *meso*-diaminopimelic acid (*m*-Dpm) at position 3 of one stem peptide and L-alanine (L-Ala) at position 4 of a second stem peptide, a configuration uncommon in gram-positive bacteria and more resembling the bacterial cell wall of gram-negative bacteria. Most gram-positive bacteria such as

Staphylococcus aureus carry L-lysine or a different amino acid at position 3 that is linked by an oligopeptide to the L-alanine at position 4 of the adjacent peptide. When a stem peptide is not cross-linked, both terminal D-Ala residues are usually cleaved off by carboxypeptidases.

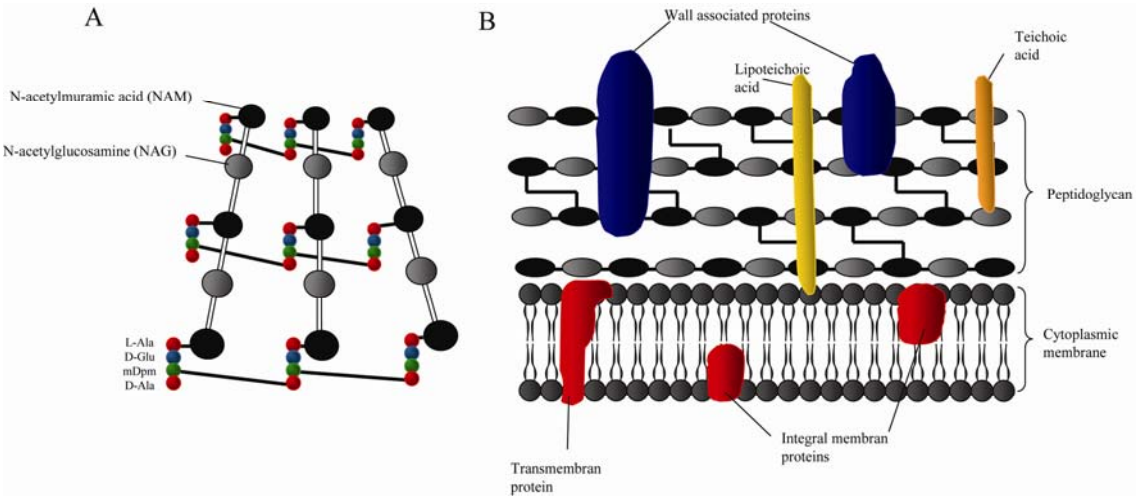


Figure 5: Cell wall of *L. monocytogenes*. (A) Network of the peptidoglycan layer. The glycan strands consist of the repeating disaccharide subunits composed of N-acetylmuramic acid (NAM) and N-acetyl-glucosamine (NAG). The peptide strands are attached to the NAM and directly cross-linked via L-Alanine (L-Ala) and *meso*-diaminopimelic acid (*m*Dpm). (B) The gram-positive cell wall is composed of a thick, multilayered peptidoglycan sheath outside of the cytoplasmic membrane. Teichoic acids are linked to and embedded in the peptidoglycan, and lipoteichoic aids extend into the cytoplasmic membrane.

In general, peptidoglycan assembly can be divided into three stages (138). The first stage of cell wall biosynthesis takes place in the cytoplasm and leads to the formation of the nucleotide sugar-linked precursors UDP-*N*-acetylmuramyl-pentapeptide (UDP-NAM-pentapeptide) and UDP-*N*-acetylglucosamine (UDP-NAG). The second stage occurs at the cytoplasmic membrane and leads to the synthesis of precursor lipid intermediates. The phospho-NAM-pentapeptide moiety of UDP-NAM-pentapeptide is transferred to the membrane acceptor bactoprenol, resulting in lipid I (NAM-pentapeptide-pyrophosphoryl-undecaprenol). The NAG from UDP-NAG is then added to lipid I, yielding lipid II (NAG-β-1,4,-NAM-pentapeptide-pyrophosphoryl-undecaprenol). The lipophilic bactoprenol plays a major role in cell wall biosynthesis as it enables the cell to transport hydrophilic precursors from the aqueous cytoplasmic environment through the hydrophobic membrane to external sites of PG incorporation. The third and final stage of cell wall biosynthesis proceeds at the

outer side of the cytoplasmic membrane, involving the polymerization of disaccharide peptide units and incorporation into the growing PG. Peptidoglycan assembly is a multistep process that involves various enzymes of the PBP (Penicillin-binding protein) family, such as transglycosylases, transpeptidases and D-alanyl-D-alanine carboxypeptidases. These enzymes synthesize cross-linked peptidoglycan from lipid intermediates and mediate the removal of D-alanine from the precursor of peptidoglycan. Five proteins have been characterized as PBPs *L. monocytogenes* (56), however more recent studies have identified six more proteins with homologies to PBP proteins (8). Although it has to maintain the turgor pressure of the bacterial cell, the murein layer is a dynamic structure that is remodeled constantly by expansion during cell growth and cleavage during cell division and lysis.

1.4. Cell wall hydrolases

The cleavage of molecular bonds in peptidoglycan is required to allow insertion of new cell wall material for both cell division and maintaining the cell shape. Murein hydrolases are enzymes that have hydrolytic activity towards certain components of the cell wall. They are present in most bacteria and yeast and play a pivotal role for cell division and lysis by cleaving peptidoglycan. They are also involved in *de novo* cell wall biosynthesis and in various cellular processes such as protein secretion, biofilm formation, genetic competence and flagella formation. Certain murein hydrolases, which are able to destroy the cell wall and therefore lead to lysis of the bacterial cell, are designated autolysins. These enzymes form holes in the peptidoglycan layer to enable incorporation of new cell wall material. Generally, they contain two different active domains, one for attachment to the cell wall and the other for cleavage activity (74). Additionally, they often possess N- or C-terminal repeat structures flanking the enzymatic domain (74). These repeats are not conserved and are probably responsible for specific binding to various components of the murein layer (53). Many autolysins have an overall basic charge at neutral pH, which may enhance their binding to negatively charged components of the cell wall. Murein hydrolases are generally translocated out of the cells via their N-terminal signal peptide. However, autolysins that are encoded by phages do not possess signal peptides and instead are secreted by cell lysis.

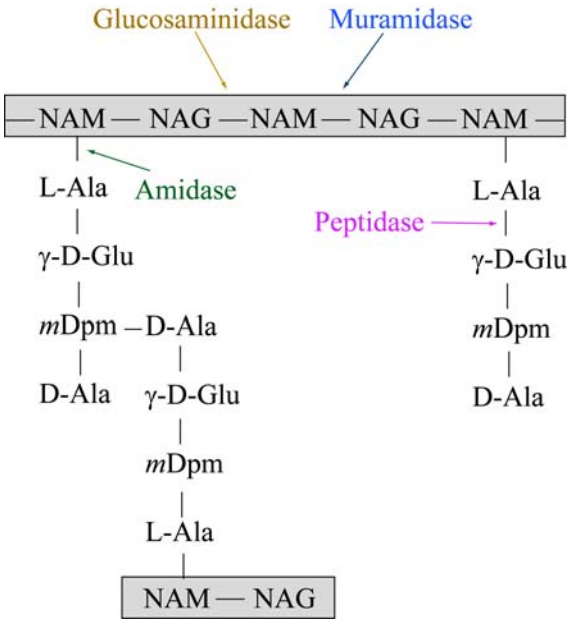


Figure 6: Cleavage sites of cell wall hydrolases in the peptidoglycan layer of *L. monocytogenes*. NAM, *N*-Acetylmuramic acid; NAG, *N*-Acetylglucosamine; L-Ala, L-alanine; γ-D-Glu, γ-D-glutamate; *m*Dpm, *meso*-diaminopimelic acid (Adapted from reference (8)).

In *L. monocytogenes*, several hydrolytic enzymes, such as p60, Ami and Auto, have been implicated in modulating virulence (103). Hydrolytic enzymes can be categorized according to their specificity of cleavage of the peptidoglycan as illustrated in Figure 6. *N*-Acetylmuramidases and *N*-Acetylglucosaminidases cleave the sugar backbone of the peptidoglycan between the alternating NAM and NAG (108). Amidases attack the amide bond between the D-lactyl group of MurNAc and the amino group of L-Ala. γ-D-glutamyl-(L)-*meso*-diaminopimelate peptidases hydrolyze the γ-D-glutamyl-(L)-*meso*-diaminopimelate linkage in the cell wall peptides.

The specificity of hydrolases can be advantagous for the bacteria, providing a strategy for survival. For example, lysostaphin, produced by the *Staphylococcus simulans* bv. Staphylocolyticus affects all *Staphylococcus* species that have pentapeptide groups with glycine (139). This gives the bacteria selective advantage in mixed populations since lysostaphin acts as a bacteriocin, killing microorganisms with a pentaglycine bridge.

1.5. Determination and maintenance of cell shape

Several cytoskeletal proteins are involved in maintaining the rod morphology of *L. monocytogenes* that is preserved by division through the same medial plane and elongation of lateral peptidoglycan (Fig. 7). The biosynthesis of cell wall in rod-shaped bacteria occurs in two steps, septation and elongation. The crucial protein for septum formation during cell division is FtsZ, a structural homologue to the eukaryotic tubulin. It forms a ring (Z-ring) in

the center of bacteria to ensure that two cells are dividing properly. Absence of FtsZ prevents separation of two dividing cells and leads to size increase and lysis. The Z-ring recruits proteins specifically required for cell division, including FtsI and FtsW that are essential for septal peptidoglycan synthesis.

In *Escherichia coli*, the *min* system consists of three proteins, MinC, MinD, and MinE that prevent cell division at the poles. MinC and D act together and are involved in positioning the midcell division site by inhibiting the formation the Z ring at the cell poles. MinE imparts topological specificity to the MinCD inhibitor complex by preventing it from working at midcell. Like *B. subtilis*, *L. monocytogenes* has clear homologues of MinC and MinD, but there is no MinE (43). The function of MinE in topological control of MinCD is provided by DivIVA, even though the strategy to fix MinCD to both cell poles is considered much simpler than the one in *E. coli*. Instead of oscillating Min proteins, the MinCD proteins of *Bacillus* are recruited to the cell poles by association with DivIVA thus resulting in a static gradient of MinCD on the membrane with a minimum at midcell (43).

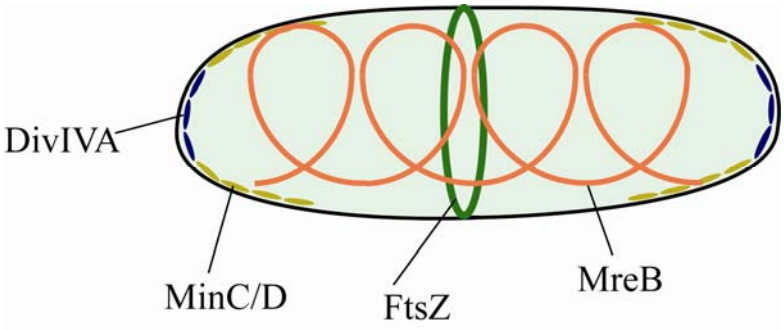


Fig. 7: Cytoskeletal proteins of rod-shaped bacteria. Virtually all eubacteria contain the tubulin-like division protein FtsZ, which forms a ring-shaped structure (Z-ring) during cell division required for the division process. The Z-ring recruits proteins that are required for septal wall synthesis and cell division. In rod-shaped cells such as *L. monocytogenes* new peptidoglycan is inserted not only at division sites during cell division but also along the sidewalls during cell elongation. The actin-like MreB protein appears as intracellular helical structure and is mandatory for rod-shaped morphology. The MinCD proteins are topologically controlled by DivIVA ensuring the placement of the division site at the midcell.

It has been shown that in most rod-shaped bacteria incorporation of new precursors into the sacculus occurs in a number of evenly distributed sites covering the cylindrical surface (36). The bacterial actin protein MreB forms helical structures along the long axis of the cell and is required for rod-shaped bacteria to make new cell wall along their length when they divide. The protein distributes various components of peptidoglycan metabolism along

the cell length. Lack of MreB leads to spherical shaped bacilli (37, 163). In *Listeria* there is an additional *mreB* homologue, *mbl* (mreB-like). Fluorescence microscopy of the *B. subtilis* MreB and Mbl proteins have shown that Mbl forms a double-helix-like structure that runs the length of the cell and MreB forms shorter helices with fewer turns within the cell (21). The close connection between cell shape and cell-wall synthesis was demonstrated for *B. subtilis* in studies by Daniel and Errington (2003). The authors made the observation that synthesis of the cylindrical part of the cell wall occurs in a helical pattern governed by Mbl (32). Another cytoskeletal protein with structural homologies to actin is ParM, which arranges an equal distribution of plasmid copies as a cell divides in two (22).

Rod-shaped gram-positive bacteria grow via to an inside-to-outside growth mechanism (162). New peptidoglycan is attached underneath the existing wall while the outermost wall layers are degraded and released into the growth medium as turnover material. Many studies involving cell wall turnover in gram-positive bacteria have been performed with *B. subtilis* (65, 126). The rate of cell wall turnover appears to correlate with the growth rate, indicating that hydrolases are more active at high growth rates (162). A direct interaction between peptidoglycan synthases and hydrolases, as has been observed in *E. coli*, appears to be unlikely in gram-positive species, because they are physically separated. While the synthases form the inner, new layers the hydrolases act well apart from the synthases on the outer layers. In most rod-shaped species, including *E. coli* and *B. subtilis*, biosynthesis of the murein layer is controlled by the MreB proteins (162).

1.6. Lipoproteins in *L. monocytogenes*

Lipoproteins have been studied extensively in gram-negative bacteria and their biosynthetic pathway was investigated in detail using Brauns' lipoprotein of *E. coli* (135). The presence of lipoproteins in gram-positive bacteria, however, has only been recognized more recently. These cell envelope components are a functionally diverse group of surface proteins that are anchored into the cell membrane by a lipidated N-terminus, thereby preventing loss into the environment. They have various important roles for the bacterial cell, including ABC transport systems, antibiotic resistance and adhesion (33), (150). In addition, lipoproteins were speculated to be involved in sensory signaling systems, immune modulation and maintenance of envelope integrity (150).

Lipoproteins are membrane-associated proteins having a consensus sequence at the C-terminal end of the signal peptide, referred to as a lipobox. It is composed of the amino

acids leucine$_{(-3)}$-alanine/serine$_{(-2)}$-alanine/glycine $_{(-1)}$ followed by a requisite conserved cysteine. The thiol group of the cysteine is lipid-modified by introducing a thioether linkage to a diacylglyceryl moiety (149), a reaction carried out by prolipoprotein diacylglyceryl transferase (Lgt), and the resulting prolipoprotein is further processed by the lipoprotein specific signal peptidase (Lsp) (135). Following cleavage of the signal peptide, the conserved cysteine residue becomes the N-terminus of the mature lipoprotein and in gram-negative bacteria the newly established N-terminal amino group is acetylated by *N*-acyltransferase (Lnt) (149). As no orthologue of this enzyme could be found in the genome of low G+C gram-positive bacteria, lipoprotein modification is considered a two step process as illustrated in Figure 8.

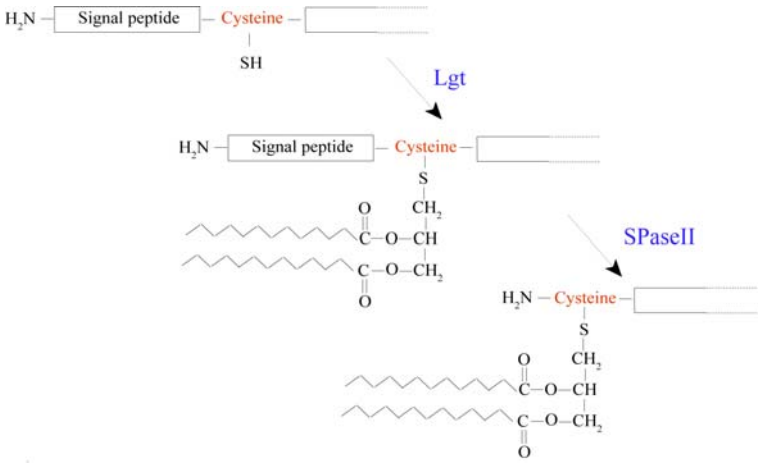


Figure 8: Biosynthesis of lipoproteins in gram-positive bacteria. Lipid modification of prolipoproteins (by thioether linkage of diglyceride to the lipobox cysteine) is followed by signal peptide cleavage, generating the mature lipid-anchored lipoprotein. (Adapted from reference (149)).

With 68 putative members, lipoproteins constitute the largest group from the 133 predicted surface proteins in *L. monocytogenes* (19, 54). Among these are 28 substrate binding components of ABC transport systems, 15 lipoproteins predicted to be involved in different enzymatic and metabolic activities and, remarkably, 25 lipoproteins of unknown function (7).

1.7. Host defense against microbes

The components of the innate immune system that discriminate between micro-organisms and self are able to recognize conserved motifs, known as pathogen-associated molecular patterns (PAMPs), found only in microorganisms. The most important PAMPs of gram-negative bacteria are lipopolysaccharides (LPS), a major component of the outer

membrane contributing greatly to the structural integrity of the bacteria and protecting the membrane from harmful chemicals. LPS is an endotoxin that can induce a strong immune response in mammals. Also, peptidoglycan serves as an excellent target for the innate immune system since it is a unique and essential component of virtually all bacterial cells and is not present in eukaryotes. The minimal biologically active moiety of bacterial peptidoglycan is muramyl dipeptide (MDP), generated by the activity of murein hydrolases. Other microbial structures that are exposed on the bacterial cell surface include lipoteichoic acids and flagellin. The innate immune system plays a central role in controlling and eliminating microbes that successfully enter an organism. Among the effector molecules of the innate immune response are antimicrobial peptides and microbial sensor molecules, such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) proteins.

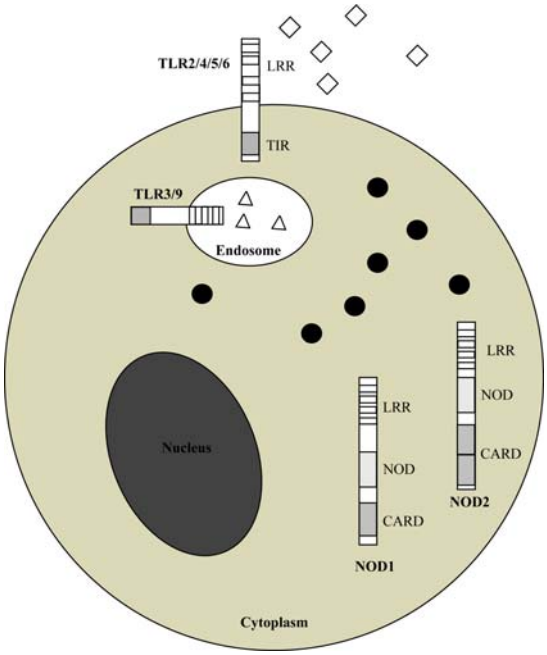


Figure 9: Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) proteins are the major host receptors for detection of microbe-derived molecules. Filled circles represent intracellular and empty squares represent extracellular PAMPs. (Adapted from reference (42)).

1.7.1. Pattern recognition receptors (PRR)

The innate immune system provides a rapid response to pathogens through primary recognition of PAMPs via ligation of signaling receptors in the host (Fig. 9). The Toll-like receptors (TLRs) have been identified as major players in the early detection of microbes. TLRs activate signal cascades and mediate the induction of nuclear factor κB (NF-κB) and interferon regulatory factor 3 (IRF3), leading to immune activation and release of pro-inflammatory cytokines. TLR family members are located either on the cell surface (e.g.

TLR4) or in endosomes (e.g. TLR9) and consist of extracellular N-terminal leucine-rich repeat motifs, followed by a cysteine-rich region, a transmembrane domain, and an intracellular Toll/IL-1 R (TIR) motif. More than 10 members of the human TLR family have been identified, having diverse yet predetermined ligand specificity such as stimulation by viral RNA or DNA and detection of bacterial components. Of particular interest to the study of bacterial pathogenesis are TLR4, which recognizes LPS, TLR5, which senses bacterial flagellin and TLR2, which was reported to detect a number of different PAMPs, including lipoteichoic acid (LTA), peptidoglycan (PG) and lipoproteins (106, 120, 141, 151). The adaptor molecule myeloid differentiation factor-88 (MyD88) is involved in TLR-dependent signal transduction by recruiting several signal components (e.g. IL1 receptor associated kinases, IRAK) leading to activation of NF-κB and to production of cytokines. TLRs detect microorganisms extracellulary or within the luminal side of the phagosome.

Another important group of microbial sensors, known as the Nod-like receptor (NLR) family, comprises cytoplasmic proteins that recognize intracellular pathogens (Fig. 9). They consist of an N-terminal effector domain important for protein-protein interaction, a centrally located nucleotide binding oligomerization domain and a C-terminal series of LRRs involved in ligand recognition and autoregulation. There are two well characterized NOD proteins, NOD1 and NOD2, that differ in their PAMP recognition. NOD1 is produced in epithelial cells of stomach and colon and in macrophages and dendritic cells of various organs (e.g. lung, kidney and spleen). It specifically detects diaminopimelic acid-containing dipeptide or tripeptide motifs present in the cell envelope of gram-negative bacteria and a few gram-positive bacteria (e.g. *L. monocytogenes*) (42). NOD2 detects the minimal biologically active moiety of peptidoglycan, muramyl dipeptide, and thus can sense peptidoglycan derived from either gram-negative or gram-positive bacteria. NOD2 has therefore been implicated as a general sensor for both gram-positive and gram-negative bacteria whereas microbe recognition by NOD1 is limited to bacteria containing *m*Dpm, mostly gram-negative bacteria. The NOD proteins are intracellular sensors and are thought to function in host defense in those tissues where TLRs are absent or expressed at low levels (121). The host must be able to discriminate between commensal organisms and microbes that pose a potential threat since PAMPs are shared between both species and constant stimulation by the normal flora has to be avoided. As such PRR expression is perhaps down-regulated and limited to certain areas of tissues or altered in distribution at the subcellular level.

1.7.2. Antimicrobial peptides

Mammals produce a variety of factors with activity against microbes that can be grouped into two different categories, the antimicrobial proteins and inorganic disinfectants (e.g. nitric oxide and hydrogen peroxide). Cationic antimicrobial peptides (CAMPs) contain some 15-45 amino acids including two or more positively charged residues and as a rule have a net positive charge. They bind electrostatically to the negatively charged bacterial surface and subsequently depolarize and permeabilized the membrane. The peptides are found in diverse organisms, including amoebae, fruit flies, plants, and mammals. They are produced in various human tissues and cell sources such as neutrophiles, monocytes, macrophages, intestinal Paneth cells and epithelial cells (86). CAMPs have an important role in innate immunity at intestinal mucosal surfaces and at the epithelium of the respiratory tract, protecting skin and epithelia against invading microorganisms and assisting neutrophils and platelets (77, 116). Among the major families of mammalian antimicrobial proteins identified are defensins and cathelicidins (41). The cathelicidins are cationic peptides with a highly conserved N-terminal structural domain, cathelin, linked to a C-terminal peptide with antimicrobial activity. LL-37/hCAP-18 is the only cathelicidin found in humans and a homologous murine peptide has been identified, referred to as cathelin-related anti-microbial peptide (CRAMP) (41). The fact that humans and mice possess only a single cathelicidin gene validates the generation of a mouse model via knock-out of Cnlp $^{-/-}$. Defensins are small (2-6 kDa) peptides with three characteristic pairs of intramolecular disulfide bonds and a β-sheet structure. Two major groups can be distinguished according to the arrangement and spacing of the disulfide bonds; namely, α-defensins found in neutrophils, macrophages, and Paneth cells in the small intestine and β-defensins found in epithelial cells.

Various bacterial pathogens (e.g. *Staphylococcus aureus* and *Salmonella enterica*) have evolved mechanisms of resistance, including modification of anionic molecules on their cellular surface to reduce the negative charge and thus repulse CAMPs (116). Among these molecules are teichoic acids, which are composed of alternating phosphate and aldiol groups that are substituted with D-alanine and *N*-acetlyglucosamine. The lipoteichoic acids (LTAs) are anchored to the cytoplasmic membrane whereas cell wall teichoic acids (TA) are linked to murein. Modification of these polymers by connecting D-alanine to the alditol residues via an ester bond, leaving a free, positively charged amino group, generates partial neutralization.
Another strategy to modify the bacterial cell surface for increased resistance to CAMPs is the lysinylation of phospholipids (152). The two free amino groups of the L-lysine moiety of

lysylphosphatidylglycerol (LPG) give the molecule a net positive charge. The two types of modifications and the effect on bacterial strains lacking enzymes required for these reactions are illustrated in Figure 10.

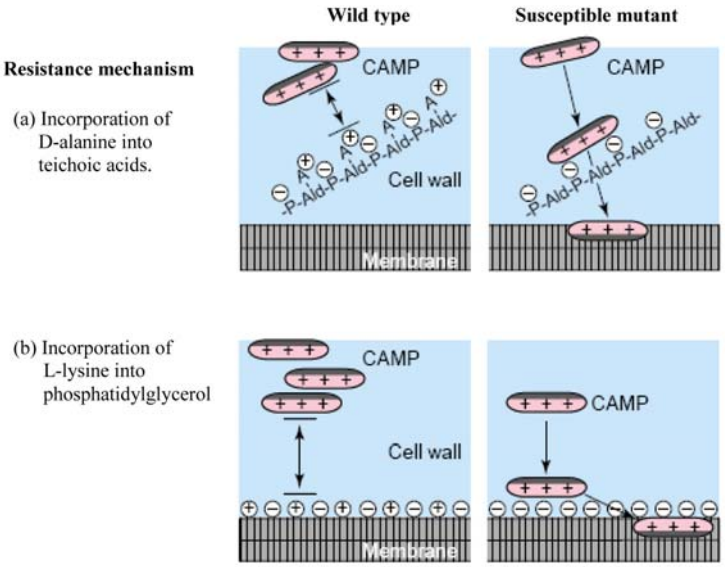


Figure 10: Proposed mechanisms of peptide resistance in gram-positive bacteria. Anionic molecules such as (a) teichoic acid polymers and (b) phosphatidylglycerol are modified in the wild-ype strain, causing repulsion of CAMPs. Mutants lack enzymes necessary for the modification, resulting in increased sensitivity to CAMPs. A, D-alanine; Ald-P, alditolphosphate. (Adapted from reference (116)).

1.8. Aims of this work

The first section of this study addresses cell wall hydrolases and their role in determining the morphology, stability and activity of the bacterial cell. Using computational analysis, surface proteins of *L. monocytogenes* were identified that carry functional domains of putative murein hydrolases and modules that are required for cell wall attachment. The properties of these enzymes were mainly investigated by characterizing deletion mutant phenotypes, including bacterial fitness and pathogenesis. The transition from rough to smooth colony morphology was studied using clinical isolates of *L. monocytogenes* with rough pheno-type. Double mutants were generated to examine the effects of concomitant lack of several murein hydrolases.

The second section deals with a recently described two-component system of *L. monocytogenes*, VirR/VirS, that has been associated with virulence. The regulation system

has been implicated in controlling the proteins involved in D-alanylation of lipoteichoic acids and MprF, which is required for lysinylation of phosphatidylglycerol. As both modification systems have been shown to add to resistance against cationic peptides, we hypothesized that VirR/VirS is a general control system for CAMP resistance. For my study, deletion mutants lacking genes encoding the two component system, *virR/virS*, and genes of the *dlt* operon were created and characterized. Also, the role of an alanine racemase encoded by the *dal* gene for modification of teichoic acids was examined by analysis of a Δ*dal* strain.

The third section of my thesis explores the relevance of lipoproteins in pathogenesis and as targets of immune recognition using a deletion mutant for the lipoprotein glycerol transferase (*lgt)*. This enzyme is required for the creation of lipoproteins by addition of the lipid moiety to the precursor form of the protein prior to cleavage by signal peptidase II. Deletion mutants of *lgt* within *L. monocytogenes* and *L. innocua* backgrounds were created, thus strains devoid of lipoproteins could be used to study TLR2 mediated immune recognition. The general role of lipoproteins for virulence and bacterial fitness was also examined using the Δ*lgt* deletion strain. Lipoproteins in *L. monocytogenes* were therefore analyzed via two major aspects, as targets of immune recognition and as virulence factors in pathogenesis.

2. Materials and Methods

2.1. Mice

Female BALB/c and C57Bl/6 mice were purchased from Harlan Winkelmann (Borchen, Germany) and used for *in vivo* infection experiments. Female TLR2$^{-/-}$ mice on a C57Bl/6 background were donated by M. Steinmueller (Medical Clinic II, Justus-Liebig-University, Giessen, Germany). All animals were kept under controlled, pathogen-free conditions. All work carried out in this study is covered by license GI15/5-26/2004 and approved by the regional board overseeing studies involving animals.

2.2. Cell culture

Cell line	Cell type	Organ	Source/reference	Catalog number
Caco2	Epithelium	Colon	Human	DSMZ ACC 169
Hela	Epithelium	Uterus	Human	DSMZ ACC 57
L-929	Fibroblast	Subcutaneous Tissue	Mouse	DSMZ ACC 2
P388D1	Macrophage	Lymphoid	Mouse	C7463 (Sigma)
PTK2	Epithelium	Kidney	Rat kangaroo	ATCC CCL-56

Growth media

MEM: Minimum Essential Medium with Earle's Salts, with L-Glutamine (Gibco BRL)

DMEM: Dulbeccos´s Modified Eagle Medium with Sodium Pyruvate, 1000 mg/l Glucose, Pyridoxin (Gibco, BRL)

RPMI 1640: with 20 mM Hepes, without L-Glutamine (Gibco BRL)

FCS: Fetal Bovine Serum (Sigma)

L-Glutamine: 100 x; 29.3 mg/ml in Normal Saline, 200 mM (PAA Laboratories)

NEA: 100 x Non-Essential Amino Acids (Seromed)

Hanks'Salt Solution: 1 x; w/o Ca^{2+}, Mg^{2+}, Phenol Red (Seromed)

Trypsin/ EDTA: 1 x; 0.5 g Trypsin (1:250)/l; 0.2 g EDTA/l in 1 x PBS (PAA Laboratories)

Freezing media: 4 ml DMSO; 78.13 g/mol (Merck) in 50 ml FCS

Cell culture dishes: Nunclon™ Δ (100 x 20 mm, Nunc), Gibson

24- Well-Plates: 24 well plates, Nunclon™

Cryo-tubes: Cryogenic Vials (Nalgene)

Freezing container: Cryo 1°C Freezing Container (Nalgene)

2.3. Antibodies

Antibody	Cell line	Source/Reference
Anti- InlA	L244C3	S. Müller, GBF Braunschweig
Anti- InlB	IC100F4	S. Müller, GBF Braunschweig
Anti- ActA	N4, N81	L. Jänsch, GBF Braunschweig
Anti- MurA	EM7G1	J. Schwarz, GBF Braunschweig
Anti- Iap	Fup 60	L. Jänsch, GBF Braunschweig
Anti-mouse IgG- Cy3		Dianova
Phalloidin oregon green		Invitrogen
Anti-mouse horse radish peroxidase		Santa Cruz

2.4. Bacterial strains

Escherichia coli

Strain	Relevant genotype	Source/reference
invα F'	*F' endA1, recA1, hsdR17 (rK, m+K), supE44, l- , thi-1 gyrA, relA1, f80, lacZa ΔM15 Δ(lacZYA –argF), deo R+*	Invitrogen
Top10	*F-, mcrA, Δ(mrr-hsdRMS-mcrBC), φ80lacZ, ΔM15 ΔlacX74, recA1, araD139, galU, galK, Δ(ara-leu) 7697, rpsL (StrR), endA1, nupG*	Invitrogen
BL21	*F-, ompT (Ion) hsdS(rB-mB-) gal dcm (DE3)*	Invitrogen

Listeria

Strain	Relevant genotype	Source/reference
L. monocytogenes EGDe	Serotype 1/2a	(92)
L. innocua	6a, CLIP11262	(52)
L. welshimeri 6b	SLCC5334	Lab strain collection

Seeliger's *Listeria* Culture Collection Mannheim

Strain	Relevant genotype	Source/reference
SLCC7500	R 1	H. Hof
SLCC7501	R 3	H. Hof
SLCC7503	R 5	H. Hof
SLCC7504	50 I	H. Hof
SLCC7506	153 IV	H. Hof
SLCC7508	1/2	H. Hof
SLCC7509	4 d	H. Hof
SLCC7510	1/2 c	H. Hof

Listeria monocytogenes EGDe

Deletion strain	Gene name	Source/reference
Δ*0129*	*lmo0129*	This study
Δ*0394*	*lmo0394*	This study
Δ*iap*	*lmo0582*	A. Amend
Δ*secA2*	*lmo0583*	(93)
Δ*secA2-secA2*	*lmo0583* complemented strain	(93)
Δ*flaA*	*lmo0690*	This study
Δ*0717*	*lmo0717*	This study
Δ*dal*	*lmo0886*	This study
Δ*dltC*	*lmo0972*	This study
Δ*dltB*	*lmo0973*	This study
Δ*aut*	*lmo1076*	This study
Δ*1104*	*lmo1104*	This study
Δ*1215*	*lmo1215*	This study
Δ*1216*	*lmo1216*	This study
Δ*1303*	*lmo1303*	This study
Δ*1521*	*lmo1521*	This study
Δ*virS*	*lmo1741*	This study
Δ*virR*	*lmo1745*	This study
Δ*1941*	*lmo1941*	This study
Δ*2203*	*lmo2203*	This study
Δ*lgt*	*lmo2482*	(6)
Δ*lgt-lgt*	*lmo2482* complemented strain	This study
Δ*spl*	*lmo2505*	This study
Δ*2522*	*lmo2522*	K. Kuchmina
Δ*ami*	*lmo2558*	This study
Δ*2591*	*lmo2591*	This study
Δ*murA*	*lmo2691*	(21)
Δ*murA* Δ*iap*	*lmo2691 lmo0582*	A. Amend
Δ*murA* Δ*aut*	*lmo2691 lmo1076*	This study
Δ*murA* Δ*ami*	*lmo2691 lmo2558*	This study
Δ*murA* Δ*spl*	*lmo2691 lmo2505*	This study
Δ*iap* Δ*aut*	*lmo0582 lmo1076*	This study
Δ*iap* Δ*ami*	*lmo0582 lmo2558*	This study
Δ*iap* Δ*spl*	*lmo0582 lmo2505*	This study

Listeria innocua

Deletion strain	Gene name	Source/reference
Δ*lgt*	*lin2625*	This study

2.5. Plasmids and Oligonucleotides

Plasmid	Description	Source/reference
pAUL-A	Temperature sensitive shuttle vector; Em^r	(23)
pAUL-A-iap	Shuttle vector with flanked iap regions	This work
pAUL-A-murA	Shuttle vector with flanked murA regions	(21)
pAUL-A-secA2	Shuttle vector with flanked secA2 regions	This work

Plasmid	Description	Source/reference
pCR® 2.1 TOPO®	TA Cloning vector for *E. coli*	Invitrogen
pPL2	*L. monocytogenes* site-specific phage integration vector	(81)
pPL2-secA2	Integration vector containing the *secA2* gene	This work
pGEX6P-1	Ap^r $lacI^q$ tac promoter	GE Healthcare
pGEX6P-1-iap	Vector carrying a 1380 bp PCR fragment of *iap*	This work
phTLR2	Human TLR2 expression plasmid	C. Kirschning
pELAM-Luc	Firefly luciferase expression plasmid	(25)
phRL-TK	Internal control reporter	Promega
pRK5	CMV-promoter	BD Pharmingen

Generation of deletion mutants

a) Listeria monocytogenes

Primer name	Sequence 5'-3'	Cut site
Lmo0129P1	AGGTTTGAGCTCGAAGTCATACTAAAATTCG	SacI
Lmo0129P2	CGCTCCTTTTTTTAGTTTTTCCTCATCCTCTCCT	N/A
Lmo0129P3	AGGATGAGGAAAAACTAAAAAAAGGAGCGGCCAA	N/A
Lmo0129P4	GAAACCGTCGACGCAGCTATCAAAGCCATCAA	SalI
Lmo0394P1	CCAGAAAAGAGCTCAGTATCTCAAGGAACGTTC	SacI
Lmo0394P2	CGCTCAGTAAAATTATCCACGCACCCCTTTTTTAT	N/A
Lmo0394P3	AAAGGGGTGCGTGGATAATTTTACTGAGCGTCACT	N/A
Lmo0394P4	CGAACAGTCGACTAAACTTAAAGATATGGTTA	SalI
Lmo0717P1	GGTATTGAGCTCGTGCTTGTTGATGGAGATGA	SacI
Lmo0717P2	GTTTATTCTCCCTTATCCACGGCACTCCTTTGTTA	N/A
Lmo0717P3	TAAAAACTACTTACGGTCTTTTTTTCT	N/A
Lmo0717P4	TGTTTCGTCGACTTTAAATTAGCAATGTTAAC	SalI
Lmo0886P1	TATGCGGAGCTCATGGACCTTCACGCCGCG	SacI
Lmo0886P2	CGAATATTACCTTCTAGATTGTTCACGTTCCTTTCT	N/A
Lmo0886P3	GGAACGTGAACAATCTAGAAGGTAATATTCGTGCTTTT	N/A
Lmo0886P4	AGCAAAGTCGACTGTCGCCATCTCTGCATAGC	SalI
Lmo0972P1	GCAAGTTCACTGGATCCTACCTAGCATATTTCG	BamHI
Lmo0972P2	CAGCTTTTTTTTCATTATAAATTACCACCTTTAGTT	N/A
Lmo0972P3	AGGTGGTAATTTATAATGAAAAAAAAGCTGTGGATGACATTTG	N/A
Lmo0972P4	GCTGCATAACGGAATTCAGGCGTG	EcoR
Lmo0973P1	GTTAGGATCCAAACTGCGCTATTATTCCG	BamHI
Lmo0973P2	CACCTTTAGTTAGTTTCACTTGTTAACCTCACTATTAAGTG	N/A
Lmo0973P3	GGTTAACAAGTGAAACTAACTAAAGGTGGTAATTTATA	N/A
Lmo0973P4	GATGAAATGGGTCGACGCGGG	SalI
Lmo1076P1	AAAGAAGAGCTCGAAAAGCAAAAACTTCCACAAG	SacI
Lmo1076P2neu	ACTTTTGCGGCCGCTTTTCCCCTCCTAAATTAA	Not I
Lmo1076P3neu	AAGCAGCGGCCGCTTAAATTATAACACTTCCATAATCTA	Not I
Lmo1076P4	TTTAAATGTCGACCTTAAACCTGCTAAAAGCAC	Sal I
Lmo1104P1	TCTCTCGAGCTCTTGCTGACACAAGAAATCT	SacI
Lmo1104P2	TTAATTCCTTTCTAAAGTATTTTACTAGCTT	N/A
Lmo1104P3	TAGTAAAATACTTTAGAAAGGAATTAATAAAA	N/A
Lmo1104P4	CGTCACGTCGACAAAGTACAGGCTCATAGCTT	SalI
Lmo1215P1	TTCGCTGAGCTCGAAAATGTTTTTCAATCTG	SacI
Lmo1215P2	TCGGTTATTTTCTAATGTCGTCACGCCTTTCTGT	N/A
Lmo1215P3	AAGGCGTGACGACATTAGAAAATAACCGAAAAAGAGCA	N/A
Lmo1215P4	GTACGCGTCGACAACAGCATCTGTTCCAACCC	Sal I
Lmo1216P1	TGATACGAGCTCAACTGGCTTTGGCAATGTG	Sac I
Lmo1216P2	TTTGCAAGGTTATTACAAATGCTTCTTTGCGAGCT	N/A
Lmo1216P3	GCAAAGAAGCATTTGTAATAACCTTGCAAAAAAAG	N/A

Primer name	Sequence 5'-3'	Cut site
Lmo1216P4	TAAGAAGTCGACGTTTTTCATTACGGTAAAATC	Sal I
Lmo1303P1	CGATAAGAGCTCTATTTACAACATTGGGAGTT	SacI
Lmo1303P2	ACTAAATCGACCTTATAATAATCCCTCCAAAAAGA	N/A
Lmo1303P3	TTGGAGGGATTATTATAAGGTCGATTTAGTGACCA	N/A
Lmo1303P4	TGAATTGTCGACGTTTGTGTTTAATACGCGAG	Sal I
Lmo1521P1	AAAAACCCGAGCTCCTTGTTATAAAAACAAGGGA	SacI
Lmo1521P2	CTAGCTTCTAATTTTTTAAAGCTAACACCACCCAT	N/A
Lmo1521P3	TGGTGTTAGCTTTAAAAAATTAGAAGCTAGAGGCG	N/A
Lmo1521P4	CACTTTATGTCGACATGTAAGTAGAGGGAAACGTCC	Sal I
Lmo1741P1	GTAAACGGATCCGGTCAGGTACTTCA	BamHI
Lmo1741P2	GGTGTACCACTTTTTAGGCGCACCTCCTCGCTACTAATTAG	N/A
Lmo1741P3	GCGAGGAGGTGCGCCTAAAAAGTGGTACACCAATTATGATTG	N/A
Lmo1741P4	CTGATATTCCGAATTCCCACAACCG	EcoRI
Lmo1745P1	CCAAGCAGGATCCGCCGATTTTTGT	BamHI
Lmo1745P4	AATACGAATTCAGCAAGTCGTTTCC	EcoRI
Lmo1745P3	AGGGAGGCACTAGAACGAAAAAACAGGGAGATAGCGCTAA	N/A
Lmo1745P2	CTCCCTGTTTTTTCGTTCTAGTGCCTCCCTCGGTGGAACT	N/A
Lmo1941P1	GTCACCGAGCTCAACTGTTGTGATTTGGATGG	SacI
Lmo1941P2	CAGTGTTGTTTATTATCAAAGTTCCTCCTTTAGTG	N/A
Lmo1941P3	AAGGAGGAACTTTGATAATAAACAACACTGAATGGG	N/A
Lmo1941P4	ATCATTGTCGACGATACCAAGATAACCAAACC	Sal I
Lmo2203P1	ATCAAAGAGCTCATATAACATGGAAGAAAGAACGAATTC	SacI
Lmo2203P2	ATTGCTACTTTTTTAATTAGTTAGTTCCCCCAATT	N/A
Lmo2203P3	GGGGAACTAACTAATTAAAAAAGTAGCAATCCAATT	N/A
Lmo2203P4	CAGAAACGTCGACCATAACTACAGCACCTGCTCC	Sal I
Lmo2505P1	TTCTTAATGAATTCCACAATCAAGATTGCAATTT	EcoRI
Lmo2505P2	TTTCAATAATAATTAAAAGTTTACCTCCTAAAAAGCTCCC	N/A
Lmo2505P3	TAGGAGGTAAACTTTTAATTATTATTGAAAAGTTAAAAAGCACC	N/A
Lmo2505P4	GTAGATTTGTCGACCTTTTAGCTTATCTTGATCATC	Sal I
Lmo2558P1	CATAATCAGAGCTCGTTCCAACCGGTAACACTCC	SacI
Lmo2558P2neu	ACTAATGCGGCCGCAGTTTAAATCCTCTCCTAAC	Not I
Lmo2558P3neu	AGTGCTGCGGCCGCATAATAAAAAACTCCCAGCCGGGTATGG	Not I
Lmo2558P4	ACCAGAAAGTCGACATAATATCATTGACTTATTACC	SalI
Lmo2591P1	TGGATCGAGCTCTGCGATTTTGATGTTTGCT	SacI
Lmo2591P2	GAACCTTTTTTTATTTACCAAATTTCAATGCACTCCTT	N/A
Lmo2591P3	TGCATTGAAATTTGGTAAATAAAAAAAGGTTCTGCATTTACG	N/A
Lmo2591P4	ATTTGACGTCGACTTAAAGCGTTTAGGGTTGG	Sal I

N/A, Not Applicable

b) Listeria innocua

Primer name	Sequence 5'-3'	Cut site
LIRDlgt 1	TTATTTGGAGCTCGAGCTGTTCGTTCC	SacI
LIRDlgt2	AAAATCTTCAACTTTAATTCCCCTACTTTCAAAAAAAGAC	N/A
LIRDlgt 3	GAAAGTAGGGGAATTAAAGTTGAAGATTTTCGAGTTTGTA	N/A
LIRDlgt4	ATCGCTGTCGACACTCCATCATACTC	SalI

N/A, Not Applicable

Complementation

Primer name	Sequence 5'-3'	Cut site
2482F-1	ATTGGGATCCTGATAAGCA	BamHI
2482R-2	CTTAATCAAACTCGAGAATCTC	XhoI

RT PCR

Primer name	Sequence 5'-3'	Cut site
0974-RT1	ACACCGGATTTCCCTTGCTA	N/A
0974-RT2	GGAGACATATGGCCGTAAACGA	N/A
1695-RT1	TTACGCATTGCCTCAGAGCAT	N/A
1695-RT2	TGTTCGCGTACTTTGACTGCTT	N/A
1741-RT1	TTTACCACGTGTTTTCGAGCAA	N/A
1741-RT2	TTCCTACGCCACTCTCAGATTG	N/A
1745-RT1	GGTGTAATCAAATCCGAGAAGTTTC	N/A
1745-RT2	TCCTCGCCATTAAAACATCCA	N/A

N/A, Not Applicable

GST protein purification

Primer name	Sequence 5'-3'	Cut site
Iap-GST-1	ATCGCATCCGCAGGATCCGTAGTAGTCGAAGCTGGT	BamHI
Iap-GST-2	GTTACTTTAAGTGAATTCTTATACGCGACCGAA	EcoRI

2.6. Growth media

SOB-Medium:
2% [w/v] Bacto Tryptone
0.5% [w/v] Bacto Yeast Extract
10 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
10 mM $MgSO_4$

BHI-Medium:
3.7% [w/v] BHI
1.5 mM Trisodium citrate
0.4 mM $MgSO_4$·
7 mM $(NH_4)_2SO_4$
0.5 M Glycerol

SOC-Medium:
2% [w/v] Bacto Tryptone
0.5% [w/v] Bacto Yeast Extract
10 mM NaC1
2.5 mM KCl
10 mM $MgCl_2$
10 mM $MgSO_4$
20 mM Glucose

2 x SMM:
1 M Sucrose
0.02 M Tris
0.01 M $MgCl_2$
0.04 M Maleic acid
pH 6.8 with 10 N NaOH

SMMP:
55 ml 2 x SMM
40 ml 4 x PAB
5 ml 5% BSA

1 M Succinic acid: pH 7.3

4 x Bacto Perassau Broth (PAB):
1.6% [w/v] Nutrient Broth (Gibco BRL)
1.4% [w/v] Bacto Tryptone (Difco)
1.24% [w/v] Yeast Extract (Gibco BRL)
pH 6.8

5 % BSA (Gerbu Fraction V): pH 7.5

DM3 Regeneration plates:
200 ml 5% [w/v] Agar
500 ml 1 M Succinic acid
100 ml 5% [w/v] Casein Hydrolysate (Gibco BRL)
60 ml 10% [w/v] Yeast Extract (Gibco BRL)
100 ml K_2HPO_4 (3.5%)/KH_2PO_4 (1.5%)
10 ml 50% [w/v] Glucose
20 ml 1 M $MgCl_2$
10 ml 5% [w/v] BSA

Fusogen:
40% [w/v] PEG 6000
50 ml 2 x SMM
q.s. 100 ml H_2O
pH 6.8
autoclaved

2.7. Antibiotics

Stock solutions:

Ampicillin	100 mg/ml in H_2O; end conc 100 µg/ml
Erythromycin	50 mg/ml in 96% [v/v] Ethanol; end conc with *Listeria*: 5 µg/ml with *E. coli*: 300 µg/ml
Penicillin	10 mg/ml in 70% [v/v] Ethanol;
Chloramphenicol	10 mg/ml in 70% [v/v] Ethanol
Gallidermin	10 mg/ml in H_2O
Colistin	35 mg/ml in H_2O
Polymyxin B	20 mg/ml in H_2O

2.8. Solutions, buffers and standards

CCMB 80:
80 mM $CaCl_2$
20 mM $MnCl_2$
10 mM $MgCl_2$
10 mM K- Acetate
10% [v/v] Glycerol
pH 6.4 with 0.1 M HCl

10 x PCR-Puffer A:
200 mM Tris·HCl (pH 8.3)
20 mM $MgCl_2$
250 mM M KCl
0.5% [v/v] Tween 20
0.1% [w/v] Gelatine

5 x Loading buffer (Agarose-Gelelektrophoresis):
25% [w/v] Ficoll Typ 400
0.25% [w/v] Bromphenol blue
in 1x TE buffer

1x TE buffer:
10 mM Tris·HCl (pH 8.0)
1 mM EDTA

50 x TAE-Puffer:
2 M Tris-HCl
17.5% [v/v] Acetic acid
0.05 M EDTA (pH 8.0)

10 x PBS:
27 mM KCl
1.4 M NaCl
81 mM Na_2HPO_4
15 mM KH_2PO_4
pH 7.4

8 x Laemli-buffer:
40% [v/v] Glycerol
2% [w/v] SDS
0.08% [v/v] ß-Mercaptoethanol
7.25 mM Tris·HCl (pH 6.8)
0.05% [w/v] Bromphenol blue

10 x SDS-Running buffer:
250 mM Tris
1.92 M Glycine
1% [w/v] SDS

Polyacrylamide Separation gel (12.5%):	Stacking gel (5.7 %):
5 ml 1.5 M Tris·HCl (pH 8.8)	2.5 ml 0.5 M Tris·HCl (pH 6.8)
8.4 ml Rotiphorese® Gel 30	1.9 ml Rotiphorese® Gel 30
0.2 ml 10% [w/v] SDS	0.1 ml 10% [w/v] SDS
10 µl TEMED	3.5 µl TEMED
150 µl 10% [w/v] APS	100 µl 10% [w/v] APS
6.25 ml H_2O	5.4 ml H_2O

Coomassie-Staining solution:
4 Tablets of Coomassieblue
10% [v/v] Acetic acid (96% [v/v])
50% [v/v] Methanol
q.s. 250 ml H_2O

Destaining buffer:
10% [v/v] Acetic acid
40% [v/v] Methanol

10 x Blot buffer (without Methanol):
0.5 M Tris
0.38 M Glycine
0.37% [w/v] SDS

1 x Blot buffer (with Methanol): 10% 10 x Blot buffer
33% [v/v] Methanol

Ponceau-S-Red- Solution: 2% [w/v] Ponceau-S- Red
30% [w/v] TCA
30% [w/v] S- Sulfoxsalicyl acid·2 H_2O

1 x TBS with Tween:
10 mM Tris-HCl, pH 8.0
150 mM NaCl
0.05% [v/v] Tween 20

1 x TBS without Tween:
10 mM Tris-HCl, pH 8.0
150 mM NaCl

Western Blot- blocking solution:
5 % [w/v] Skim milk powder in 1 x TBS without Tween

DNA-Standard
1kb-Ladder (Gibco BRL): 12.2, 11.2, 10.2, 9.2, 8.1, 7.1, 6.1, 5.1, 4.1, 3.1, 2.0, 1.6, 1.0, 0.5, 0.4, 0.34, 0.3, 0.22, 0.2, 0.16, 0.13, 0.08

Protein-Standard:
Prestained See Blue™ (Anamed): 250, 98, 64, 50, 32, 25, 16, 6, 4 kDa

2.9. Equipment

Machine	Source
Agarose electrophoresis chamber	Self-construction of institute
Analytical balance	Mettler H54
Autoclave	Getinge
Bacterial culture incubator	Heraeus
Balances	Kern, EG
Blotting chamber	Biometra
Bioanalyzer	Agilent 2100
Cell culture incubator	Forma Scientific, Inkubator 3250
Clean bench	Heraeus, HERA safe
Cooling centrifuge	Heraeus, Megafuge 1,0 R
Digital camera	Canon
Dispenser	Brand, Dispensette
Ice machine	Icematic F90 Compact Electronic
Fluorescence microscope	Zeiss, Axiophot
Freezer	Heraeus; Bosch
Gel documentary system	Cybertech CS1, Cybertech
Lab centrifuge	Heraeus, Biofuge 15
Light table	Ewem- Janus
L-max plate reader	Molecular Devices
Magnet stirrer	Ikamag, RCT
Microscope	Zeiss, Standard 25
Microwave	Moulinex
Mini centrifuge	Heraeus pico, Biofuge 15
Multi- pipet	Matrix, Independent 6-Kanal
PH-Meter	Knick

Machine	Source
Photometer	Pharmacia, Ultrospec 3000
Pipets	Eppendorf
Protein gel electrophoresis chamber	Biometra
Real-time PCR	ABI Prism 7700 sequence detection system
Shaker	Infors; GFL-3017
Scanner	Epson Stylus Color 600
Speedvac- concentrator	Bachofer
Thermoblock	Self-construction of institute
Thermocycler	Perkin Elmer, GeneAmp PCR System 2400
Thermomixer	Eppendorf, Comfort
Video copy processor	P68E, Mitsubishi Electric
Voltage power supply	Pharmacia MultiDrive XL, Consort E455 and E865
Vortex mixer	Janke & Kunkel, Ika Vibrofix VF1
Water bath	Lauda
Water bath shaker	GFL 1083; HT

2.10. Bacterial cultures

2.10.1. Cultivation of strains

Strains were grown on agar plates at 37°C and stored at 4°C for 4 to 8 weeks. *E. coli* strains were maintained on LB-plates and *Listeria* strains on BHI-plates, supplemented with appropriate antibiotics when necessary. For long-term storage 0.75 ml of bacterial overnight cultures were mixed with 0.75 ml sterile 60% [v/v] glycerine in BHI, shock-frozen with liquid nitrogen and stored at -80°C. Bacterial cultures were grown over night at 37°C in 100 ml flasks at 10 or 20 ml LB broth for *E. coli* or BHI broth for *Listeria* and generally diluted 1:50 the next day for fresh cultures.

2.10.2. Measurement of bacterial growth

The optical density of cell suspensions was determined with a spectral photometer (Ultrospec 3000, Amersham Pharmacia Biotech) in microcuvettes of 1 cm width at 600 nm. The samples were diluted 1:10 with suspension media when OD_{600}-Values exceeded 0.6. Automated measuring was performed using the Infinite 200 plate reader (Tecan) in 96-well plates with 200 µl volume/well.

2.10.3. Autolysis assay

For Triton X-100 induced autolysis bacterial cultures were grown to an OD_{620} of 0.3 and immediately chilled on ice. Cells were harvested by centrifugation at 10,000 × *g* at 4°C for 5 min and washed with ice-cold H_2O. The pellet was resuspended in 50 mM glycine- 0.01% triton X-100 buffer (pH 8.0) and to obtain a suspension of OD_{620} von 1.0. The samples were

shaken at 37°C and 200 rpm and optical density was measured spectrophotometrically. The rate of autolysis was determined with the decrease of optical density.

2.10.4. Antibiogram

To determine changes of deletion mutants in sensitivity against antibiotics 50 µl of bacterial overnight cultures were mixed 1:1 with BHI broth and the suspension was plated on BHI agar plates. Filter plates (Roche) containing antibiotics were stamped onto the plates and areas of growth inhibition were measured after 20 h of incubation at 37°C. According to inhibition areas strains were categorized into resistant, intermediate and sensitive.

2.10.5. Swarming motility assay

To examine flagellar motility bacterial cultures are stabbed into BHI soft agar plates (0.3%) and the extent of spreading is measured on day 5 of incubation at 20°C. The magnitude of spreading is compared to that of wild type to assess a relative rate of motility.

2.11.6. Biofilm assay

Bacteria were cultured in 200 µl of BHI or LB- medium in 96 well plates for 1 day at 37°C or 4 days at 20°C without shaking. Media was removed and wells were washed in three times in PBS. Sessile bacteria were stained with 0.01 % crystal violet for 1 h on the tumbler and the stain was removed. Cells were washed three times in PBS and the stain was resolved in 100% ethanol for 1 h on tumbler. The optical density was measured at 550 nm or 595 nm and values were normalized to wells of the EGDe wild type.

2.11. DNA and genetic manipulations

2.11.1. Chromosomal DNA isolation

Chromosomal DNA was isolated from *Listeria monocytogenes* following the protocol of Pitcher *et al*. (124); Bacterial cells from 1.5 ml overnight grown culture were harvested by centrifugation for 5 min at 1,500 × *g*, washed with 0.85% (w/v) NaCl solution resuspended in 100 µl TE buffer containing 50 mg/ml lysozyme. The samples were incubated at 37°C and 1,200 rpm (Thermomixer) for 30 minutes and 1 µl DNAse-free RNAse (Boehringer Mannheim) was added to the pellet and incubated for 5 min at room temperature (RT). 500 µl GES-reagent were added and the reaction tube was inverted 5 times followed by 5 min

incubation at RT and 2 min on ice. Finally, 250 µl ice-cold 7.5 M ammonium acetate were added for protein precipitation and tubes were incubated on ice for 10 min followed mixing by inverting the sample 5 times. The DNA was extracted by adding 500 µl phenol/chloroform / isoamyl alcohol (25:24:1) to the reaction tube and rotating the samples for 15 min. After a 15 min centrifugation step at 16,000 × *g* and 4°C the upper aqueous layer was removed with a pipette and placed in a new microfuge tube. DNA was precipitated by adding 0.5 volumes of 2-propanol followed by initially careful and finally vigorous shaking and centrifuged at 16,000 × *g* for 5 min at 4°C. After 5 washing steps with 70% ethanol the DNA was air-dried and resuspended in 100 µl H_2O. The DNA pellet was dissolved by incubating the samples overnight at RT and stored at 4°C the next day.

2.11.2. Plasmid isolation and purification

Isolation of plasmid DNA from *E. coli* was performed either with GFX™*Micro* Plasmid Prep Kit (Amersham) for small scale preparation or with JETstar 2.0 Plasmid MIDI Kit (Genomed) for big scale preparation. The DNA was eluted with H_2O (50 µl or 500 µl) and stored at -20°C. DNA was purified after PCR or enzymatic reactions with the Qiaquick PCR Purification Kit (Qiagen) and eluted in 5 mM Tris (pH 8.5). DNA extraction from agarose gels were performed after enzymatic digestion of inserts from plasmid DNA. For that purpose the kits Gel extraction (Qiagen) and QiaEX II agarose gel extraction (Qiagen) were used and DNA was eluted in 20-30 µl H_2O.

2.11.3. Agarose gel electrophoresis

Separation of DNA molecules between 0.2 kb and 20 kb is carried out with agarose gels of different concentrations in the electric field. As standard, a 1% agarose gel containing 50 µg ethidium bromide (Roth) was used in TAE buffer. 25% Ficoll, stained with bromphenol blue, was used as loading buffer and the 1 kb DNA LADDER™ (Life Technologies) as size marker. In most cases gels were run at 150 V, 500 mA for 40 min and DNA was visualized with UV light (254 nm) using the gel documentation system.

2.11.4. Enzymatic modification of DNA

For enzymatic cleavage of Plasmid DNA or of PCR products generally 1 µg of DNA were incubated in a reaction volume of 50 µl for 3-4 h with appropriate restriction endonucleases (5-25 units, Fermentas) at 37C°. When necessary, plasmid DNA was dephosphorylated before ligation to prevent relegation of the vector. In this reaction 1 U Alkaline Phosphatase

(Calf Intestine Phosphatase, 1 U/µl; Boehringer Mannheim) were used for 0.2 to 2 µg DNA in a 30 µl reaction at 37°C for 1 h. The enzyme was deactivated after addition of 3 µl 200 mM EGTA at 65°C for 10 min. For ligation of insert and plasmid DNA 1 µl (1 U/ µl) of T4-Ligase (Life Technologies) was used in a 20 µl reaction at 14°C for 15 h or at 22°C for 1.5 h.

2.11.5. Cell transformation

Chemical transformation of *E.coli*- according to Hanahan (59). The invαF´ *E. coli* strain was grown over night in SOB media at 30°C and diluted the next day 1:50 with 50 ml SOB media. After shaking at 30°C 200 rpm for 2-3 h the OD_{550} of about 0.3 was reached and the culture was transferred into a sterile 50 ml tube and incubated on ice for 10 min. Cells were harvested by centrifugation at 4,000 × *g* and 4°C for 15 min and pellet was resuspended in 17 ml CCMB80. The suspension was incubated on ice for 20 min and cells were pelleted and resuspended in 4.3 ml CCMB80. The homogenous mix was transferred in 200 µl aliquots to chilled 1.5 ml cryo-tubes and stored at -80°C after shock-freezing with liquid nitrogen. The bacterial cells were thawed on ice and mixed with the ligation reaction or with 50 ng clean plasmid. The mix was incubated on ice for 30 min and the heat pulse occurred for 90 sec at 42°C followed by 2 min incubation on ice. The cell suspension was mixed with 800 µl SOC-media, transferred to a sterile 12 ml tube and incubated for bacterial regeneration at 37°C for 1-3 h at 180 rpm. For selection the cultures were plated on LB agar plates containing the appropriate antibiotics in volumes between 50 to 200 µl and incubated over night at 37°C.

Electroporation of Listeria according to Park and Stewart (115). *Listeria* were grown over night in BHI broth and diluted the next day 1:100 in 200 ml BHI broth containing 0.5 M sucrose. After the OD_{600} of 0.2 was reached 2 mg penicillin was added and the culture was incubated for another 2 h at 37°C and 180 rpm. The suspension was transferred to four sterile 50 ml tubes and cells were harvested by centrifugation at 5,000 × *g* and 4°C for 10 min. The pellet was resuspended on ice in 5 ml cold 1 mM HEPES- buffer (pH 7.0) supplemented with 0.5 M sucrose. After three washing steps with the same buffer cells were resuspended in 0.5 ml HEPES- buffer supplemented with 0.5 M sucrose and 10% [v/v] glycerine. Aliquots of 50 µl were transferred to chilled 1.5 ml cryogenic tubes and stored at -80°C after shock freezing with liquid nitrogen. The samples were thawed on ice, 1 to 2 µg of plasmid DNA was added and the mix was transferred to chilled electroporation cuvettes. The electroporation was performed at 1.0 kV, 400 Ohm und 25 µF and 800 µl BHI + 0.5 M sucrose were added. The

mix was transferred into sterile 12 ml tubes, incubated for 1-3 h at 30°C or 37°C and plated onto BHI agar plates supplemented with selective antibiotics.

Chemical transformation of *Listeria* according to Wuenscher (169). *Listeria* were grown over night in 0.2% glycine in BHI, diluted 1:100 in 30 ml BHI and grown to an OD_{600} of 0.6-0.8 at 37°C and 180 rpm. 25 ml of the culture were transferred to a sterile 50 ml tube and centrifuged at 5,000 × *g* for 10 min. The pellet was washed in 25 ml H_2O, resuspended in 2.5 ml SMMP and transferred to a sterile 50 ml tube. 250 µl of a lysozyme solution (100 mg/ml) in 2 x SMM was added and the mix was incubated for 15 h at 37 °C. The protoplasts were centrifuged at 5,000 × *g* for 10 min, washed in 10 ml SMMP and carefully resuspended in 2.5 ml SMMP. Aliquots of 300 µl were transferred to sterile 1.5 ml cryo-vials and stored at -80°C after shock-freezing with liquid nitrogen. The samples were thawed on ice and transferred to 15 ml tubes and carefully mixed with 0.5-1 µg plasmid DNA. Slowly, 2 ml of Fusogen were added and the mix was shaken in circles for 1 min, followed by 1 min incubation at 22°C and addition of 7 ml SMMP. The suspension was inverted 5 times, centrifuged at 5,000 × *g* for 20 min and the supernatant was removed carefully with a pipet. Protoplasts were resuspended 1 ml SMMP and shaken for 3 hours at 30° C. The culture was plated at 333 µl on DM3- plates with the appropriate antibiotics and incubated for 4-5 days at 30° C.

2.11.6. Polymerase chain reaction (PCR)

For screening purposes *Taq*-Polymerase (Life Technologies) was used. Colony material from agar plates was used as a template and the following reaction was prepared:

	50 µl
10 x PCR-Puffer A	5.0 µl
5 mM dNTPs	2.0 µl
20 µM Primer forward	0.5 µl
20 µM Primer reverse	0.5 µl
Taq-Polymerase (5 U/µl)	0.1 µl
H_2O	42 µl

For cloning purposes polymerases with proof-reading activity were used, such as the *Pfu* – Polymerase (Invitrogen) and the Expand High Fidelity PCR Systems (Boehringer Mannheim). Chromosomal DNA was isolated from *Listeria* strains according to Pitcher (124) and used as template for cloning reactions. The dNTP stock solution was 200 µM (from 100

mM dNTP Set, Life Technologies). Oligonucleotides were used as 20 pmol/µl stock solution for all PCR reactions. The following conditions were used for standard PCR screen:

Step of reaction	Temperature	Time	Cycle
Initial denaturation	95°C	5 min	1 x
Denaturation	95°C	30 sec	
Primer annealing	50-58°C	30 sec	35 to 50 x
Primer extension	72°C	1-3 min	
Final extension	72°C	7 min	1 x
Cooling	10°C	∞	

2.11.7. Generation of deletion mutants

The flanking regions of the gene of interest was PCR-amplified using oligonucleotide primer pairs 1+2 and 3+4 designed so that the ends of the resultant PCR products contain complementary sequences. The two DNA molecules were joined in a second PCR with primers 1+4 and the amplified product was cloned into the temperature sensitive vector pAUL-A restriction enzymes. Plasmid DNA was extracted from positive clones of invαF' cells and sequencing of inserted DNA was performed by the sequencing service of the institute. The vector was then electroporated into *Listeria monocytogenes* EGDe, followed by incubation at 30°C. Integration of the plasmid into chromosomal DNA by homologous recombination was carried out with a temperature shift to 42°C. Successful integration was determined by the disappearance of a band in PCR using vector-specific oligonucleotides. Excision of the plasmid together with the gene of interest was carried out in the following step. Bacteria were grown in BHI at 30°C for 12 h and the culture was diluted 1:100 in BHI and grown at 30°C to an OD_{600} of 0.3-0.5. Then the culture was diluted 1:20 in BHI containing 10 µg/ml erythromycin. The culture was incubated for another 2 h at 30°C, supplemented with 200 µg/ml ampicillin and shaken over night at 30°C. During this step erythromycin resistant bacteria carrying the plasmid are replicating and killed by the ampicillin. Next day, the culture was plated on BHI-Agar plates in serial dilutions and incubated at 30°C. Clones were transferred onto Em-selection plates and Em-sensitive clones were tested for deletion with PCR analysis.

2.12. Microscopy

For microscopic images of bacterial colonies, the various strains were grown on BHI agar plates at 37°C (supplemented with 8 µg/ml chloramphenicol as needed) and resultant

colonies were analyzed at 50 × original magnification. For visualization of bacterial cells, overnight cultures grown in BHI broth were washed in PBS, Gram-stained and fixed onto objective slides for microscopic analysis at 200 × or at 1,000 × original magnification (Axiophot, Zeiss). For electron microscopy, 10 ml bacterial cultures were grown to early log phase ($OD_{600} \approx 0.3$) and the cells fixed by adding 1.3 ml formaldehyde, incubating for 5 min at 25°C then adding 0.8 ml glutaraldehyde followed by an additional 2 hour incubation on ice. Cells were harvested by centrifugation (6,200 × *g* at 4°C for 15 min) and the pellet resuspended in 2 ml of 1% formaldehyde cacodylate buffer (cacodylate buffer, 0.1 M cacodylate, 0.09 M sucrose, 0.01 M $MgCl_2$, 0.01 M $CaCl_2$, pH 6.9). The samples were sent to Manfred Rohde (GBF, Braunschweig) for transmission electron microscopy.

2.13. RNA

2.13.1. RNA isolation and purification

Bacterial cultures in BHI were grown to exponential phase and 3 samples of 0.5 ml were prepared with 1 ml RNA protect reagent (QIAGEN). After 5 min incubation at room temperature cells were harvested by centrifugation at 13,000 × *g* for 3 min and stored at -80°C. Total bacterial RNA was extracted using the RNeasy mini kit (QIAGEN) after a wash in SET buffer (50 mM NaCl, 5 mM EDTA, and 30 mM Tris-HCl pH 7.0) containing 10% sodium dodecyl sulfate and a pretreatment at 37°C for 30 min with shaking at 350 rpm with 0.1 ml of Tris-HCl pH 6.5 containing 50 mg/ml of lysozyme, 25 U of mutanolysin, 40 U of SUPERase, and 0.2 mg of proteinase K (Ambion) and finally treated with 4 U of DNase I (RNase free; Ambion). The total RNA was quantified by absorbance at 260 and 280 nm, and quality was analyzed using the Agilent 2100 bioanalyzer.

2.13.2. Quantitative PCR analysis

Quantitative real-time PCR was performed on the ABI Prism 7700 sequence detection system. Forward and reverse primers (Table S1 in the supplemental material) were designed and purchased from Operon Biotechnologies to produce an amplicon length of about 150 bp. Quantitative PCR was performed for 40 cycles with 2.5 µl of cDNA, 25 µl of 2xSYBR green PCR master mix (Applied Biosystems), and 10 pM (each) forward and reverse primers in a final volume of 50 µl. A standard curve was generated for each primer pair by using different copy numbers of genomic DNA from *L. monocytogenes* EGDe. For each primer pair, a

negative control (water), an RNA sample without reverse transcriptase (to determine genomic DNA contamination) were included as controls during cDNA quantification.

2.14. Cell culture and virulence studies

2.14.1. Infection assay

The infection studies were performed in 24 well plates. Invasion of bacteria in epithelial cells was examined using Hela and Caco2 cells with exponentially grown bacteria. The bacterial intracellular growth was determined in P388D1 macrophages with overnight cultures of the bacterial strains. Before infection bacteria were cultured in BHI at standard conditions and harvested by centrifugation at 8,000 × *g* for 2 min. The pellet was resuspended in proper cell culture media and the suspension was used for infection at a multiplicity of infection (MOI) of 100. Eukaryotic cells were infected and incubated for 1 h at 37°C. Media was replaced with the same media containing 50 µg/ml gentamicin after 3 wash steps with PBS and cells were incubated for 1 h, 3 h, 5 h or 7 h. Cells were washed in PBS, lysed in cold, sterile 0.2% Triton X-100 and diluted samples were plated on BHI agar plates. After incubation at 37°C for ~12 h colonies were counted and numbers were compared referred to EGDe wild type.

2.14.2. Luciferase reporter assay

NF-κB activation was determined by performing a luciferase reporter assay with Hela cells transiently expressing human TLR2 as well as the reporter plasmids pELAM-Luc and phRL-TK. Confluent overnight cultures of Hela cells in 6-well plates, grown in DMEM supplemented with 10% fetal calf serum (FCS) and 1x Penicillin/Streptomycin (Invitrogen), were washed in DMEM and transfected with a mix of 4.8 µg of plasmid DNA and 15.5 µl Lipofectamine 2000 (Invitrogen). After 5 h, medium was replaced with DMEM supplemented with 10% FCS and after an additional 2 hours, the cells were split, transferred to 96-well plates at 4×10^4/well and incubated overnight in DMEM supplemented with 5% FCS. *Listeria* was grown to exponential phase to obtain a suspension of 3×10^7 CFU/ml, harvested by centrifugation, resuspended in DMEM containing 1% FCS and 20 µl aliquots added to each well for an MOI of 15. For stimulation with culture supernatants, bacteria were grown at 37°C in BHI for 12 h, clear supernatant collected by centrifugation at 5,000 × *g* for 10 min and 20 µl aliquots added to each well. Control samples were treated with an equal volume of sterile BHI medium. For treatment with pneumococcal lipoprotein lipase (Sigma), 100 µl

supernatant samples were incubated with increasing amounts of enzyme for 30 min at 37°C, followed by inactivation at 72°C for 20 min and 20 µl aliquots added to the transfected Hela cells. Stimulated cells were incubated for 5 h in DMEM supplemented with 1% FCS, washed with PBS, lysed with 20 µl Passive Lysis Buffer (Promega) and stored at -20°C. Measurement of NF-κB-mediated luciferase activity was carried out with the Dual-Luciferase Reporter Assay System (Promega) using an L-max plate reader. Signal measurements were performed in triplicate and experiments were repeated at least three times. The primary firefly luciferase signal was normalized to the signal of *Renilla* luciferase in each well, resulting in relative luciferase activity. Results are presented as fold changes of stimulated activity to non-stimulated activity.

2.15. Proteins

2.15.1. Isolation of proteins from *L. monocytogenes*

Method (I)(105)

The subcellular fractionation procedure was as described by Monk and colleagues (72, 105) with slight modifications. Briefly, 50 ml cultures of *L. monocytogenes* were grown in BHI at 37°C to log phase (OD_{600} ≈ 1.0) and the cells were subsequently harvested by centrifugation at 6,200 × *g* for 15 min at 4°C. Culture supernatant proteins were precipitated on ice overnight by treatment with 10% trichloroacetic acid (TCA), pelleted by centrifugation at 4°C, washed with ice-cold acetone, resuspended in 0.2 ml 1 M Tris-HCl pH 8.8 and stored at -20°C. For sub-cellular protein fractions, the cell pellet was resuspended in 1 ml wash buffer (10 mM Tris-HCl pH 6.9, 10 mM $MgCl_2$) containing 500 mM sucrose (SWB) and washed twice to remove traces of the supernatant. Protoplasts were generated by incubating the cells at 37°C for 2 hours in 0.1 ml SWB containing 10 mg/ml lysozyme (Merck), 2500 U/ml mutanolysin (Sigma) and 1 mM phenylmethylsulfonyl fluoride (Sigma). The cell wall fraction was obtained by centrifugation (6,200 × *g* for 15 min at 4°C) and transfer of the supernatant to a fresh tube. Protoplasts were washed in 1 ml SWB, resuspended in 0.2 ml protoplast lysis buffer (PLB; 100 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 362 U Benzonase® Nuclease (Sigma)), frozen at -20°C and lysed via three freeze-thaw cycles. The cytoplasmic fraction was obtained by centrifugation (16,000 × *g* for 10 min at 4°C) and transfer of the supernatant to a fresh tube. The resulting pellet containing the cell membrane fraction was washed in 1 ml PLB and resuspended in 0.1 ml TE buffer (10 mM Tris-HCl pH

8, 1 mM EDTA). The amount of protein in the various sub-cellular extracts was determined using a Bradford assay (BioRad) and the fractions were stored at -20°C.

Method (II) (39).

Another method to obtain cell wall–associated or cytoplasmic protein fractions was performed as described below. Briefly, 30 ml cultures of *L. monocytogenes* were grown in BHI at 37°C to log phase ($OD_{600} \approx 1.0$) and the cells were subsequently harvested by centrifugation at 6,200 × *g* for 15 min at 4°C. The pellet was resuspended in 1% (w/v) SDS in PBS and incubated for 45 min at 180 rpm and 37°C. After another centrifugation step at 6200 × *g* for 15 min at 4°C soluble proteins were separated from cells and precipitated in 10% TCA at 4°C for 12-24 h. Protein was spun down, air dried and dissolved in 100 ml 1 M Tris/HCl (pH 8.8). The centrifuged cells were washed with H_20, resuspended in 10 mg/ml lysozyme in SMMP broth and incubated for 12 h at 37°C. Protoblasts were washed once in SMMP broth, lysed with 100 µl cold H_2O and released cytoplasmic proteins were obtained in the supernatant by centrifugation at 20,000 × *g* and 4°C.

2.15.2. Immunodetection and comparative analysis of proteins

Proteins were separated by 12.5% SDS-PAGE, transferred to nitrocellulose membranes and probed with monoclonal antibodies against the protein of interest. Detection was performed using horseradish peroxidase (Santa Cruz) coupled anti-mouse or anti-rabbit antibodies and the ECL kit (GE Healthcare) as per the manufacturer's instructions. Alternatively, proteins were detected with alkaline phosphatase conjugated anti-mouse or anti-rabbit antibodies and BCIP (5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt). For comparative analysis, relative protein concentrations were derived by scanning and quantifying both Coomassie Blue-stained SDS-PAGE gels and immunoblots. Relative protein expression was estimated by extrapolating the relative protein concentration upon normalizing to the constitutive expression levels of two or more unrelated proteins and was processed using the Image J software package (129).

2.15.3. Detection of lytic activity with cell wall substrates in SDS-polyacrylamide gels

Cell wall substrate for *L. monocytogenes* or *B. subtilis* was isolated by harvesting bacterial cultures and rupture of cells in PBS using the Fast Prep 24 system (MP) with Lysing Matrix B. Cell wall material was boiled in 8% SDS for 10 h, washed three times in PBS and

resuspended in PBS. Lytic activity was detected using the protocol of de Jonge and colleagues (34). Briefly, proteins are resolved using 12.5% SDS-polyacrylamide gels containing 0.2% (w/v) autoclaved and lyophilized *Micrococcus lysodeikticus* ATCC 4698 cells (Sigma). After electrophoresis, proteins are renatured in 25 mM Tris-HCl pH 7 containing 1% (v/v) Triton X-100 until lytic bands are visible. Visualization of bands is enhanced by staining with 1% (w/v) methylene blue (Sigma) in 0.01% (w/v) KOH and subsequent destaining with distilled water.

2.15.4. Production of recombinant GST-p60 and GST-MurA fusion proteins

The coding region of *iap* (excluding the signal peptide) was amplified by PCR using the Iap-GSTfor/Iap-GSTrev primer pair and *Listeria monocytogenes* EGDe as template. The product was digested with BamHI-EcoRI and inserted into the corresponding sites of the GST gene fusion vector pGEX6P-1 (GE Healthcare). The GST-MurA fusion construct was derived from previous studies (24). The MurA and p60-GST fusion proteins were overexpressed in *E.coli* BL21 transformed with the pGEX6P-1-*murA* or pGEX6P-1-*iap* construct, and purified using the Glutathione Sepharose 4B Batch/Column method (GE Healthcare). PreScission Protease (GE Healthcare) was used to remove the GST tag from the recombinant p60 protein and purity was assessed by SDS-PAGE. The eluted protein was stored at -20°C after addition of glycerol to 10%.

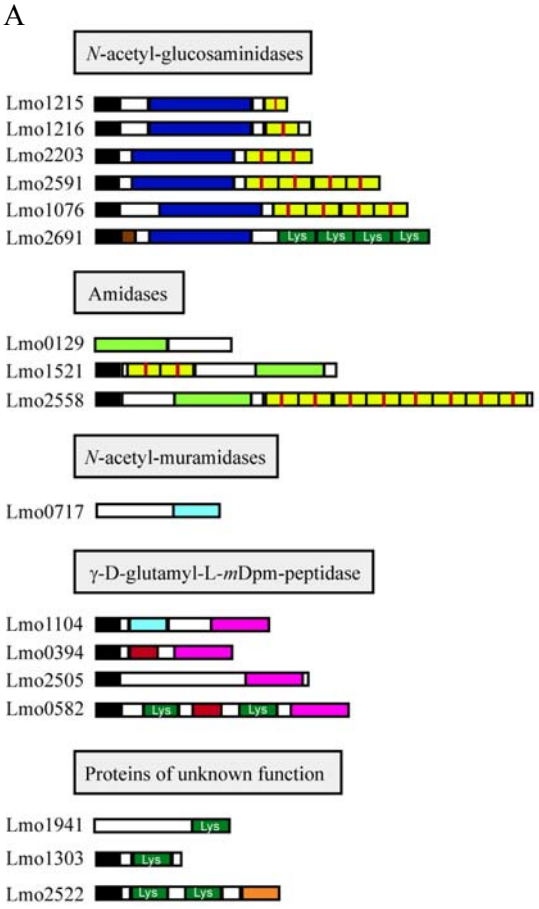
3. Results

3.1. Cell wall hydrolases in *L. monocytogenes*

3.1.1. Identification of putative murein hydrolases in *L. monocytogenes*

The importance of autolysins in maintaining normal bacterial cell functions is suggested by their widespread occurrence. Previous studies demonstrated that cell wall hydrolases are not only involved in the degradation of bacterial cell wall but also in virulence of *Listeria*. The murein hydrolases p60 (*iap*), Ami (*ami*) and Auto (*aut*) have been shown to be critical for host invasion and infection in mouse models (20, 103, 122). As such, autolysins can be considered virulence factors enabling the bacteria to enter the host successfully. Additionally, the effect of autolysins on host immune responses has been investigated. Cell wall degradation products can be recognized by pattern recognition receptors thus murein hydrolases potently modulate inflammatory and innate immune responses. Recently, Humann *et al.* (2006) have shown that a lack of the p60 hydrolase leads to decreased production of proinflammatory cytokines, such as IFN-γ, IL12p-70 and IL6 (66), and to reduced NK cell activation. The importance of autolysins for host entry and stimulation of immune responses by generating bioactive muropeptides makes them interesting targets for studying bacteria-host interactions. In *L. monocytogenes* two of the most prominent autolysins found by zymogram analysis are p60 and MurA (*murA*). Theses murein hydrolases have been characterized previously as important factors for bacterial cell division (24, 122). The proteins carry specific motifs that are required for cell wall binding and enzymatic activity. We sought to find other proteins of *L. monocytogenes* with murein hydrolase activity by scanning for domain homology to previously characterized autolysins such as MurA and p60. Using comparative genome analysis we were able to identify the genes listed in Figure 11 as candidates for deletion studies to characterize their role and function in *L. monocytogenes*. The following sections provide details on how the proteins were grouped based on the various domains involved in cell wall attachment and degradation (8).

A



B

Lmo number	Gene name	Motif	Predicted function
1215		GW	Glucosaminidase
1216		GW	Glucosaminidase
2203		GW	Glucosaminidase
2591		GW	Glucosaminidase
1076	*aut*	GW	Glucosaminidase
2691	*murA*	LysM	Glucosaminidase
0129			Amidase
1521		GW	Amidase
2558	*ami*	GW	Amidase
0717		LT	Muramidase
1104		LT,60	Peptidase
0394		p60	Peptidase
2505	*spl*	p60	Peptidase
0582	*iap*	LysM, p60	Peptidase
1303		LysM	Unknown
1941		LysM	Unknown
2522		LysM	Unknown

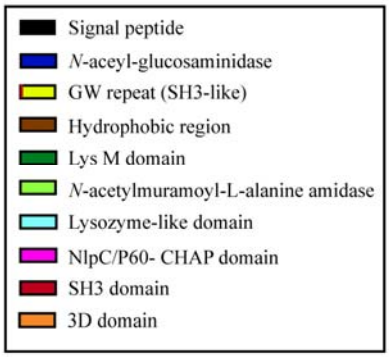


Figure 11: Surface proteins involved in cell wall hydrolysis in *L. monocytogenes*. **(A)** Schematic representation of possible murein hydrolases with the functional domains obtained by computational analysis of sequence homologies. **(B)** List of enzymes in *L. monocytogenes* involved in cell wall hydrolysis and their predicted functions. (Adapted from reference (8)).

GW modules. There are several possibilities for proteins to be linked to the bacterial surface. In particular, GW modules and LysM motifs (see below) provide non-covalent interactions with the peptidoglycan layer for various murein hydrolases. GW modules are tandem repeats that begin with the dipeptide Gly-Trp. The first characterized protein of this type in *L. monocytogenes* is InlB comprising three GW motifs of ~ 80 amino acids. The modules promotes the linkage of InlB to the bacterial surface by binding LTAs but can also interact with eukaryotic molecules, for example glycosaminoglycans (GAGs) and gC1q-R, a binding protein for the globular head domains of complement component C1q (73, 98). The motifs are involved in cell wall binding of staphylococcal murein hydrolases like AtlC of *S. caprae* and AltE of *S. epidermidis* (8). GW domains of 20 amino acids with only weak similarity to GW listerial modules have been described for LytA and PspA of *Streptococcus*

pneumoniae and for CspA of *Clostridium acetobutylicium* (8). These modules bind to choline residues of teichoic acids and lipoteichoic acids. In addition to the previously described hydrolases Ami and Auto, we found GW motifs present in Lmo1215, Lmo1216, Lmo1521, Lmo2203 and Lmo2591.

SH3 domains. GW repeats contain Src homology 3 domains that are often indicative of proteins involved in signal transduction related to cytoskeletal organization. The region bound by the SH3 domain is in all cases proline-rich and contains PXXP as a core-conserved binding motif. The function of the SH3 domain is not well understood although they may mediate many diverse processes such as increasing local concentration of proteins, altering their subcellular location and mediating the assembly of large multiprotein complexes. In *Staphylococcus simulans* the bacteriocin lysostaphin contains an SH3 homologous region (Pfam PF08460) that mediates binding to the cell wall (3). Two p60 proteins of *L. monocytogenes*, encoded by *iap* and *lmo0394*, have an SH3 domain (Pfam PF08239) independent of the GW module.

LysM motifs. LysM motifs are composed of ~ 40 amino acids and are found in various proteins having enzymatic activities related to cell wall degradation or binding (6). They domains are thought to be general peptidoglycan binding modules (8), and most LysM containing proteins are bacterial murein hydrolases (18). Generally, LysM domains of glycosylases, such as muramidases and glucosaminidases, are located downstream of the active-site domain at the C-terminus of the protein. In contrast, PG endopeptidases harbor LysM domains in the N-terminal part upstream of the active site. The different topology might be needed for proper positioning of the active-site domains towards their specific substrates (18). Also, the number of LysM motifs has been shown to vary considerably among PG hydrolases. The C-terminal LysM domain of AcmA, the major autolysin of *Lactococcus lactis*, binds only to specific sites on the bacterial surface. The presence of specific lipoteichoic acids hinder AcmA binding and hence binding only occurs near the poles and the septum of the cells. Some bacterial LysM proteins such as staphylococcal immunoglobulin G (IgG) binding proteins and intimin of *Escherichia coli* (8) have also been linked to pathogenesis. LysM motifs (Pfam PF01476) are found in six listerial proteins including MurA and p60 that carry four and two of these domains, respectively. Among the listerial proteins newly identified as having LysM motifs are Lmo1303, Lmo1941 and Lmo2522.

Peptidases. An important group of *L. monocytogenes* murein hydrolases is the p60 protein family. These proteins have a common C-terminal NlpC/p60 domain (Pfam PF00877)

consisting of 100 to 110 amino acids and are related to the CHAP (cysteine histidine-dependent amidohydrolase/peptidase) domain (8). Members of the p60 family in *L. monocytogenes* include p60, p45 (*spl*), Lmo0394 and Lmo1104 and they are predicted to be involved in peptidoglycan hydrolysis (8). Lmo1104 is found only in *L. monocytogenes* whereas the other p60 proteins have orthologues in other listerial species. P60-like proteins with predicted cell wall hydrolase function also exist in other gram-positive bacteria, such as *Bacillus halodurans, B. subtilis* and *S. aureus* (19).

Muramidases. In addition to the C-terminal NlpC/p60 domain, Lmo1104 also carries the N-terminal lytic *N*-acetyl-muramidase domain (Pfam PF01464) and may therefore have a dual function in hydrolysing the cell wall. The *N*-acetyl-muramidase motif is a lysozyme-like domain, which is also found in Lmo0717.

Glucosaminidases. Six proteins, among which are the LysM protein MurA and the GW protein Auto, carry a domain with similarities to the catalytic domain of *N*-acetyl-glucosaminidases (PF01832). Auto is the only protein of this subgroup that is absent in other *Listeria ssp.*.

Amidases. Putative amidases domains (PF01520) were found in two GW proteins, Ami and Lmo1521, and Lmo0129, a protein lacking modules known to target the bacterial cell wall.

3D domain. Protein Lmo2522 harbors a domain with 3 conserved aspartate residues, known as the name '3D'-domain. This conservation is suggestive of a cation binding function and the motif has been shown to be part of a β-barrel domain of MltA, a lytic transglycosylase of *E.coli*.

3.1.2. Smooth-rough transition in *L. monocytogenes*

Diverse studies of gram-positive and gram-negative bacteria have demonstrated that deletions of genes encoding murein hydrolases produce defects in cell morphology and cell wall synthesis, leading to the generation of long multiseptate filaments (cell chains with cross walls between individual cells). Simultaneous inactivation of several autolysins in *B. subtilis* is concomitant with the formation of longer chains of cells (70, 96, 97, 113). In *Streptococcus pneumoniae*, depletion of PcsB, an essential putative murein hydrolase, elicits the generation of long cell chains (110). Deletion of multiple murein hydrolases in *E. coli* results in the formation of filaments (64). The occurrence of bacterial cell chaining has also been observed with *L. monocytogenes*, presenting a 'rough' colony morphology initially described in 1979

by Zachar and Savage (170). In later studies rough colony isolates were shown to present greater colonization of stainless steel surfaces (105). The authors suggested that the morphologic conversion from rough to smooth might be a strategy to improve bacterial colonization of gastrointestinal tissues.

3.1.2.1. Morphology of rough variants in *L. monocytogenes*

Wild type colonies of *L. monocytogenes* exhibit smooth edges and are built mainly of single, rod-shaped cells (Fig. 12, panels 1a and 1b). In contrast, the rough phenotype is characterized by formation of cell filaments and the change in cell morphology from 'shiny and even' to 'dull and jagged'. We obtained clinical isolates (H. Hof, Mannheim, Germany) of *L. monocytogenes* that, by repeated passage on blood agar plates, gave rise to variants with rough colony morphology on agar plates (Fig. 12, panels 2a to 9a) and long filaments in microscopic analysis (Fig. 12, panels 2b to 9b). Rough isolates varied in the colony-jaggedness of edge formation and in the length of filaments (e.g. compare SLCC7501 to SLCC7506 in Fig. 12).

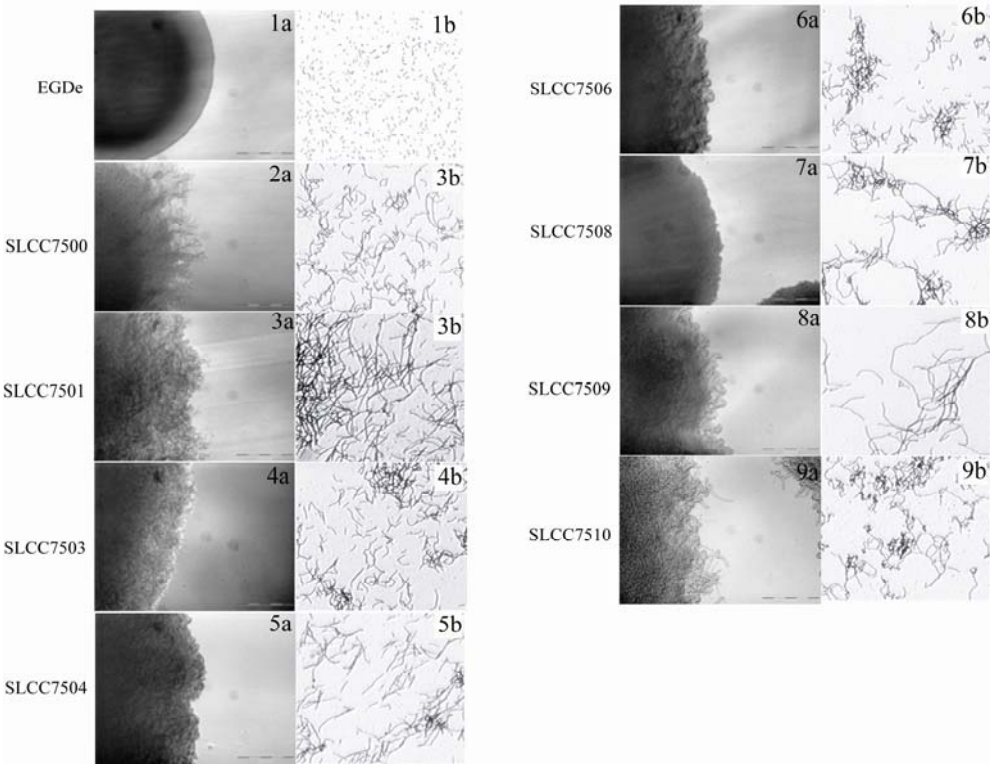


Figure 12: Microscopic morphology of the wild type *L. monocytogenes* EGDe and rough isolates. (a) The edges of colonies of the various strains grown on BHI agar plates at 37°C were compared by microscopic analysis (50 × original magnification). The wild type has a smooth outline whereas the R variants have jagged edges. Bars, 500 μm. (b) Images of individual cells of wild type and R variants are shown (200 original magnification). Bacteria were grown overnight in BHI broth at 37°C followed by Gram staining. All rough isolates exhibit long filaments in contrast to the small individual cells of EGDe.

3.1.2.2. Expression of P60 and MurA in rough strains

A defect in murein hydrolase activity was considered to be one of the possible causes for the transition of smooth to rough morphology. Thus, the rough strains were used to test whether there are certain gene products responsible for the rough phenotype that are common in these isolates. The strains were examined by immunoblot analysis for their ability to express the two autolysins, p60 and MurA. Protein extracts were isolated from the supernatant, cell wall, membrane, and cytoplasm of cultures of the wild type and rough mutants of *L. monocytogenes* grown to exponential phase. A Western blot assay was carried out with the monoclonal Fup60 mouse antibody, which is highly specific for the 60-kDa p60 protein.

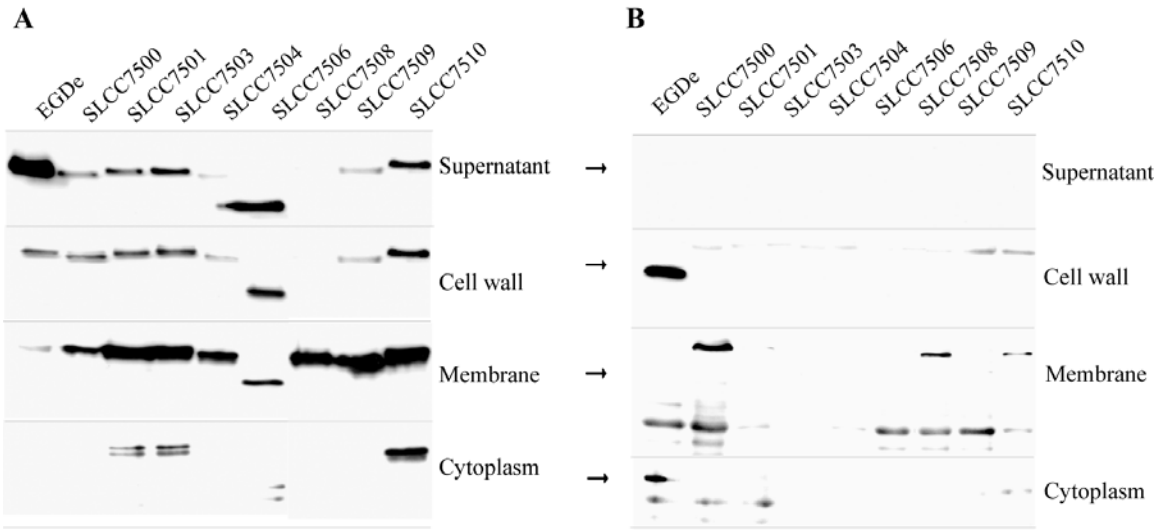


Figure 13: Immunoblot analysis quantifying MurA and p60 from *L. monocytogenes* EGDe wild type and rough variants. Proteins were isolated from culture supernatant, cell wall, membrane, and cytoplasm. Mouse monoclonal antibody Fup60 was used to detect p60 **(A)** and mouse monoclonal antibody p70128G4 was used to detect MurA **(B)** in protein extracts from *L. monocytogenes* EGDe wild type and rough mutants. Arrows indicate MurA protein.

Seven of eight mutants displayed a decreased level of extracellular p60 (from 2% to 30% of wild type level) while one strain, SLCC7506, showed a moderate level (60%) of a truncated protein in the supernatant (Fig. 13A). Those strains with diminished extracellular p60 exhibited a cellular relocation of the protein, which accumulated in the membrane fraction (3- to 12-fold more than wild type protein). Most strains also showed increased levels of p60 in the cytoplasm (e.g. SLCC7501 and SLCC7503, with three- and six-fold more protein, respectively). In contrast, no obvious difference from wild type was seen in the cell wall fraction. Western blot assays of the MurA protein were performed using the mouse

antibody p70128G4. The prominent, marked band in Figure 13B is the MurA protein with a molecular mass of 66 kDa, whereas the upper and lower bands in the membrane fraction are from nonspecific antibody binding. For all strains, the MurA protein could not be detected in the membrane fraction, as only nonspecific bands were visible. In wild type EGDe MurA is mainly located in the cell wall, however, it is not observed in the rough variants. To a lesser extent the protein was also detected in the cytoplasmic fraction of the wild type, but not of the rough strains (Fig. 13B).

3.1.2.3. Truncation of P60 results in a rough phenotype

The *iap* gene of the rough strain SLCC7506, with a truncated p60 having a level of expression comparable to that of wild type, was sequenced to determine the nature of the mutation. The protein carries a number of single, nonsense mutations and has an internal 7-amino-acid deletion (accession number AM040043) (Fig. 14). A stop codon at position 375 truncates the protein by 100 amino acids in the region comprising the NlpC/p60 catalytic domain. Deletion of this region thus appears to inactivate p60, generating the same phenotype as that for mutants that lack p60 expression altogether.

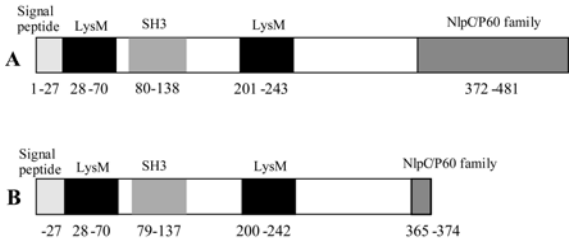


Figure 14: Nonsense mutations of the truncated p60 in SLCC7506. Diagram of the precursor protein with its known functional motifs encoded by *L. monocytogenes* EGDe **(A)** and the R variant SLCC7506 **(B)**.

3.1.2.4. Murein hydrolase activity in smooth and rough strains of *L. monocytogenes*

The activity of murein hydrolases in rough *L. monocytogenes* strains was determined by a zymogram analysis. Protein extracts from supernatant, cell wall, membrane and cytoplasm were isolated to assay for lytic activity. The zymogram revealed a large variation in the lytic profile of the different strains for the supernatant extracts (Fig. 15). The strains SLCC 7504, SLCC 7506 and SLCC 7509 showed increased lytic activity of secreted proteins compared to wild type. At the same time these three strains presented a reduced number of lytic bands by analysis of surface-associated and cytoplasmic proteins. The other rough

strains displayed fewer bands of active hydrolases in all protein fractions with respect to the parental strain.

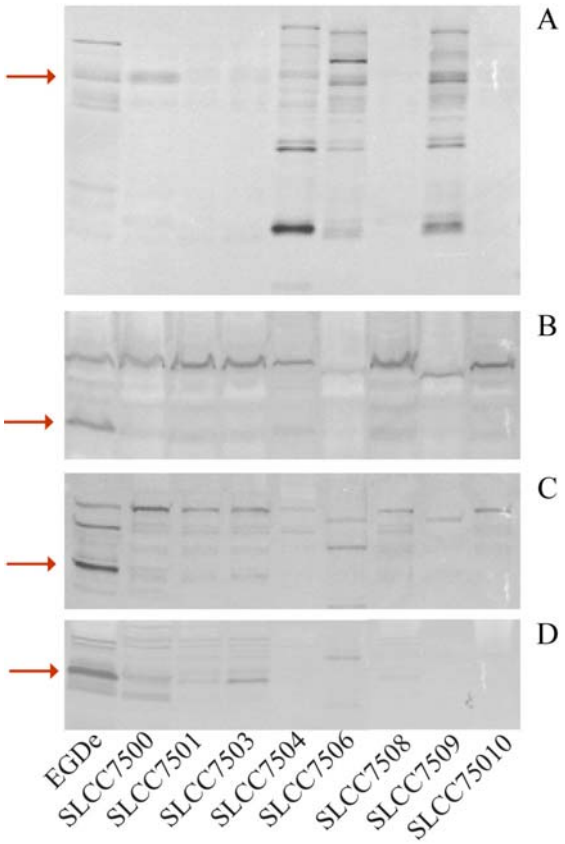


Figure 15: Zymographic analysis of *L. monocytogenes* rough variants and deletion mutants. Proteins extracts of **(A)** culture supernatant, **(B)** membrane, **(C)** cytoplasm and **(D)** cell walls were prepared and proteins were applied to an 12.5% SDS-polyacrylamide gel containing 0.2% lyophilized *Micrococcus lysodeikticus* ATCC 4698 cells. Separated proteins were renatured with 25 mM Tris-HCl (pH 7) containing 1% Triton X-100, and lytic bands were visualized by staining with 1% methylene blue in 0.01% KOH followed by destaining with water. Arrows indicate MurA protein.

3.1.2.5. Biofilm formation

In the environment, microorganisms can either exist as planktonic cells or as communities in biofilms attached to a surface and enclosed in a matrix. The surfaces colonized by microbial biofilms are mainly medical devices, water pipes, industrial equipment and food processing facilities (50). The potential for biofilm formation in the food industry is a serious concern, in particular for foodborne pathogens including *L. monocytogenes*, *Bacillus cereus* and *S. aureus*. We therefore investigated the ability of rough *Listeria* strains to form biofilms on plastic surfaces. Bacterial cultures were grown in 96-well plastic plates at 37°C without shaking and wells were washed with PBS. Sessile cells were stained with crystal violet and absorption was measured at 595 nm. For all rough strains, a decrease in biofilm formation (< 60%) during sessile growth was observed in comparison to EGDe (Fig. 16).

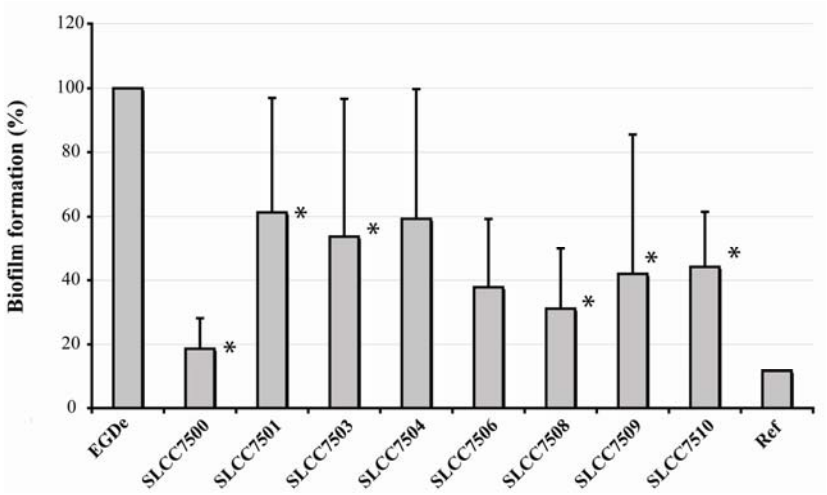


Figure 16: Biofilm formation of smooth and rough *Listeria* strains. Bacterial cultures were grown in 96-well plates for 12 h in BHI at 37°C without shaking. Sessile bacteria were washed in PBS and stained with crystal violet and absorption was measured at 595 nm. * $p < 0.05$

3.1.3. Analysis of deletion mutants lacking *iap*, *murA* or *secA2*

3.1.3.1. Production and affinity purification of recombinant p60 and MurA protein from *E. coli*

For analysis of the two autolysins MurA and p60, recombinant proteins were cloned and over-expressed in *E. coli* strain BL21(DE3). A *murA-gst* fusion construct in vector pGEX6P1 was used from a previous study (24) to express recombinant MurA. For expression of p60, the *iap* gene was amplified using primers Iap-GST-1 and Iap-GST-2 and cloned into pGEX6P1. GST-fusion protein expression was induced by IPTG, proteins were extracted by sonication and purification was performed with the glutathione Sepharose 4B Batch/Column method. Figure 17A and B (panels a) illustrate the purity of the proteins a various steps as noted. The GST tag was cleaved using Prescission Protease and purity of cleaved protein was assessed using monoclonal antibodies against MurA, p60, and GST, respectively (Fig. 17A and B, panels b). The activity of the murein hydrolases was tested by zymogram analysis using *Micrococcus* or *Bacillus* substrate (Fig. 17A and B, panels c). The MurA protein showed strong activity in lytic gels with both substrates. However, p60 had only weak lytic activity using *Bacillus* substrate and almost no activity with *Micrococcus* substrate.

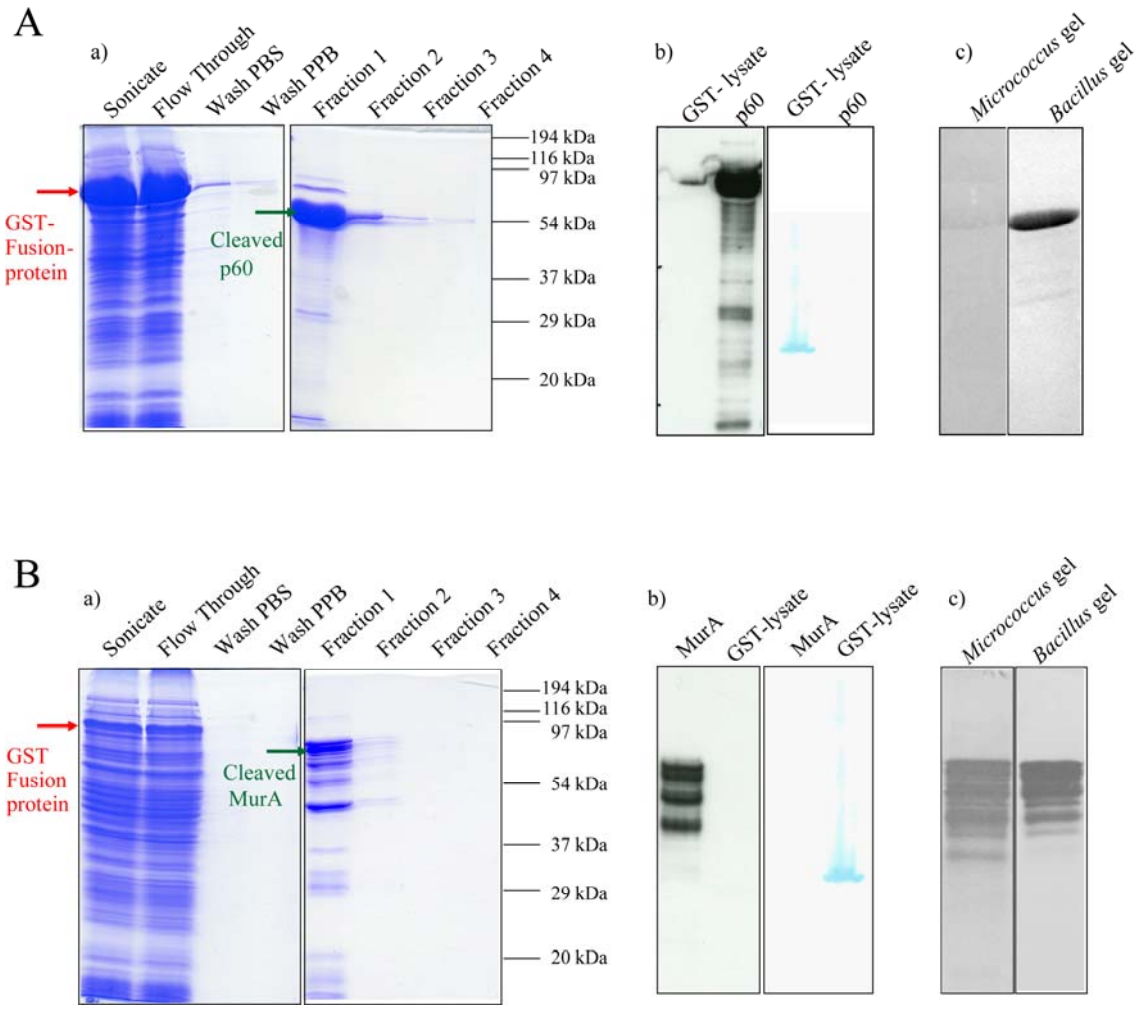


Figure 17: Purification and activity of p60 and MurA recombinant proteins expressed in *E.coli*. The expression of the glutathione S-transferase (GST) fusion constructs with **(A)** *iap* and **(B)** *murA* was induced with IPTG and proteins were extracted from the *E.coli* strain BL21 by sonication. The purification of the GST fusion proteins was carried out with the the glutathione Sepharose 4B Batch/Column method as shown in panels (a). Purity of the cleaved protein was examined by immunobloting using specific anti p60 and MurA monoclonal antibodies linked to horse-radish peroxidase and anti GST antibody linked to alkaline phosphatase (panels b). Activity of the proteins was tested by zymogram analysis using *Micrococcus* and *Bacillus* substrate (panels c).

3.1.3.2. Characterization of Δ*iap*, Δ*murA*, Δ*iap* Δ*murA* and Δ*secA2* deletion mutants and complemented strains.

In *L. monocytogenes*, the 60-kDa secreted autolysin p60 (82) is known to be involved in cell division and the generation of long filaments and is associated with invasion into certain mammalian cell lines. Mutants impaired in the synthesis of p60 were shown to be strongly attenuated in a mouse model of infection (82, 122). The protein was characterized as

a murein hydrolase based on homology to a repeat domain of an autolysin in *Enterococcus faecium* (46) and the observation that p60 overexpression induces autolysis in *L. monocytogenes* (168). In various spontaneously occurring phenotypically rough mutants of *L. monocytogenes*, decreased production of extracellular p60 has been observed (75, 82, 168).

To determine the gene products required for the smooth-rough transition, we utilized previously described individual mutants of *L. monocytogenes* EGDe lacking the genes for *iap*, *murA*, and the Δ*murA* Δ*iap* double mutant (24, 93). In addition, an isogenic Δ*secA2* deletion strain was generated, that was reported by Lenz and Portnoy (89) to cause smooth-rough transition in *L. monocytogenes*. As the SecA2 secretion system has been shown to be responsible for the transport of both of the autolysins p60 and MurA, we used the Δ*secA2* strain for phenotypic comparisons. Given difficulties both in cloning the *iap* gene and with transformation into Δ*iap* strains, complementation of the Δ*iap* and Δ*murA* Δ*iap* mutants was performed by treatment with exogenous recombinant MurA (24) and p60 protein. The complementation of the Δ*secA2* mutant was carried out using the *L. monocytogenes* site-specific phage integration vector pPL2 harboring *secA2* (136). The phenotypes of *L. monocytogenes* EGDe and deletion mutants were analyzed by light and electron microscopy. For comparisons, different strains were grown overnight in BHI broth at 37°C to early exponential phase. Despite defects in cell division, the growth behavior of the deletion mutants in BHI broth at either 20°C or 37°C did not differ substantially from that of the parental strain (data not shown). The Δ*murA* and Δ*iap* single mutants formed smooth and shiny wild type-like colonies on BHI agar plates (compare panels 1a, 2a, and 3a in Fig. 18A).

However, Δ*murA* Δ*iap* and Δ*secA2* strains lacking both extracellular murein hydrolases displayed rough colony morphology (Fig. 18A, panel 4a; 5B, panel 1a). The deletion of *murA* and *iap* could be complemented by treatment with exogenous recombinant proteins MurA and p60, thus restoring wild type-like cell morphology (Fig. 18A, panels 2b to 4b and 2c to 4c). For the Δ*secA2* mutant, complement strains were created with the EGDe *secA2* gene (Δ*secA2-C*) as well as with the *secA2* gene of several of the rough variants (Fig. 18B, panels 2 and 3). Microscopic analysis revealed that the double mutant as well as the single Δ*secA2* deletion mutant causes a rough phenotype with filaments greater than 10 μm in length (Fig. 18C, panels 4a and 5a). Both mutants lack indentations between individual cells but carry septa that separate one cell from another, as verified by transmission electron microscopy (Fig. 18C, panels 4b and 5b). In contrast, deletion of the *iap* gene alone in *L. monocytogenes* resulted in significantly shorter cell chains with an average length of 3 μm

(Fig. 18C, panel 3a) and did not cause smooth-to-rough transition as reported earlier (27). Analogous to the double mutant the filamentous phenotype of the Δ*iap* strain showed no distinct partition between individual cells (Fig. 18C, panel 3b).

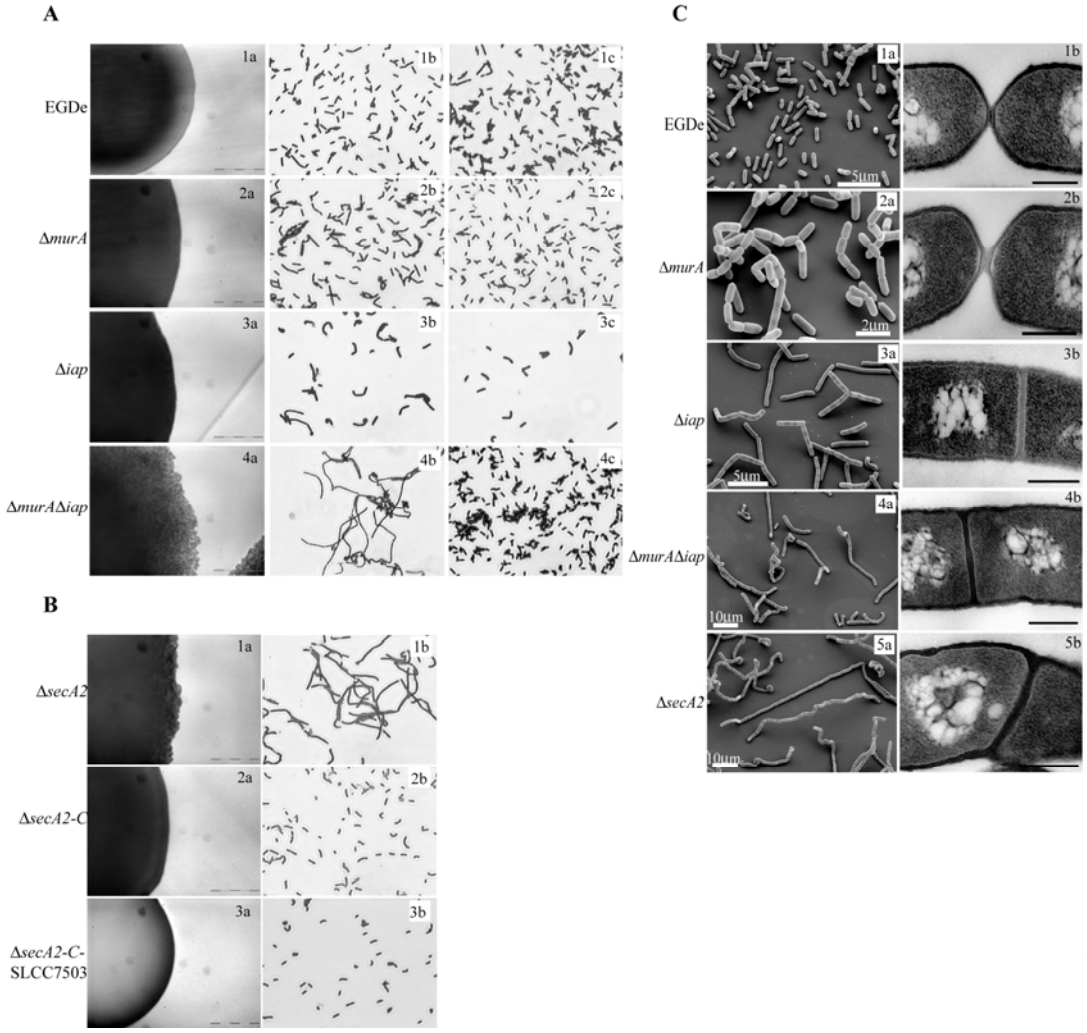


Figure 18: Microscopic analysis of the cell morphology of *L. monocytogenes* EGDe, deletion mutants and complemented strains. **(A)** Images of a colony (a) and cells (b) of EGDe and *murA* and *iap* single and double mutant strains are shown. (c) Complementation of *murA*, *iap*, and *murA iap* strains by treatment with recombinant proteins MurA and p60. EGDe serves as a control for protein treatment. Pictures in panels b and c were taken from cells in early log phase at 1,000 × original magnification. Bars in panel a, 500 μm. **(B)** Images of a colony (a) and cells (b) of the *secA2* strain and its *secA2-C* and *secA2-C*-SLCC7503 complement strains. Bars in panel a, 500 μm. **(C)** Scanning (a) and transmission electron (b) microscopy of wild type and deletion mutants. For electron microscopy, bacterial cells were harvested at an OD_{600} of 0.3 and prepared for further analysis. Bars in panel b, 0.25 μm.

The Δ*murA* mutant, on the other hand, formed long chains only in exponential phase but not in late lag phase, in agreement with the studies by Carroll *et al.* (24). In late lag phase there were mostly single cells (data not shown), whereas in early exponential phase the cells formed chains (Fig. 18C, panels 2a and 2b) that unlike the filaments of the rough strains

showed a clear contraction between each single bacterial cell. Complementation of the *secA2* gene in the Δ*secA2* deletion mutant restored the smooth phenotype and allowed for growth of individual cells.

3.1.4. A secondary *secA* gene and a homologous *murA* gene are present in the apathogenic species *L. innocua* and *L. welshimeri*

Through sequence analysis of other *Listeria* strains a secondary *secA* gene (accession number AM040040) was identified in *L. innocua* and *L. welshimeri*. The SecA2 transport systems reported to date have been found almost exclusively in pathogenic bacterial strains (90). The presence of active MurA has so far only been reported for *L. monocytogenes* (24).

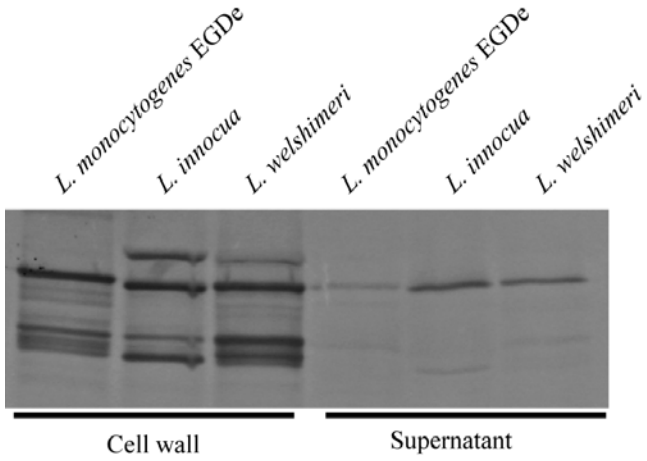


Figure 19: Activity assay for MurA in *L. monocytogenes* EGDe, *L. innocua* and *L. welshimeri*. Cell wall associated proteins of the *Listeria spp* were separated by electrophoresis in an SDS-gel containing 0.2% lyophilized *Micrococcus lysodeikticus* ATCC 4698 cells. Renaturing and visualization of proteins were performed as described in materials and methods.

Our analysis revealed that homologous *murA* genes (accession number AM039955) also exist in the chromosomal DNA of *L. innocua* and *L. welshimeri* (Table 2). An alignment of the amino acid sequences indicated a high degree of similarity between the MurA proteins of these species. It was therefore interesting to determine whether these MurA homologues have the same activity as the corresponding *L. monocytogenes* protein. To this end, supernatant and cell wall protein extracts from cultures of *L. monocytogenes* EGDe, *L. innocua*, and *L. welshimeri* were isolated to assay for lytic activity. All strains investigated here showed a prominent lytic band in the fraction associated with cell wall (Fig. 19). Thus, *Listeria* species other than *L. monocytogenes* express a functional, homologous MurA protein.

3.1.5. Analysis of deletion mutants Δ*ami*, Δ*aut*, Δ*spl* and of combinatory deletion mutants with Δ*iap* and Δ*murA*

3.1.5.1. Generation of single-and double- deletion mutants for the murein hydrolases Ami, Auto and p45

Since the simultaneous deficiency of two murein hydrolases resulted in a severe phenotypic change, it was of interest to assess the effects of a combinatory deletion of *murA* and *iap* with other published cell wall hydrolases. Ami, Auto and p45 are three characterized cell wall hydrolases in *L. monocytogenes* (20, 103, 140) with domain similarities to p60 and MurA. To compare their role with the previously analyzed strains, the deletion mutants Δ*ami*, Δ*aut* and Δ*spl* were created via a homologous recombination method (see Appendix A). In addition, the double mutants Δ*murA* Δ*ami*, Δ*murA* Δ*aut*, Δ*murA* Δ*spl* and Δ*iap* Δ*ami*, Δ*iap* Δ*aut*, Δ*iap* Δ*spl* were generated. Briefly, the suicide vector pAUL-A, carrying flanking sequences for the genes of interest, was transformed into *L. monocytogenes* EGDe, Δ*murA* or Δ*iap* strains, respectively, and the integrants (Em^r) were confirmed by PCR (Apendix A). Plasmid excision was monitored by selection of erythromycin-sensitive clones and gene deletion was verified by the PCR. Deleted chromosomal regions of interest were sequenced to demonstrate the internal in-frame deletion.

3.1.5.2. Morphology of newly generated single and double mutants

The bacterial strains were grown to exponential phase (OD_{600} of ~ 1.0) and Gram-stained to determine the cell shape via light microscopy.

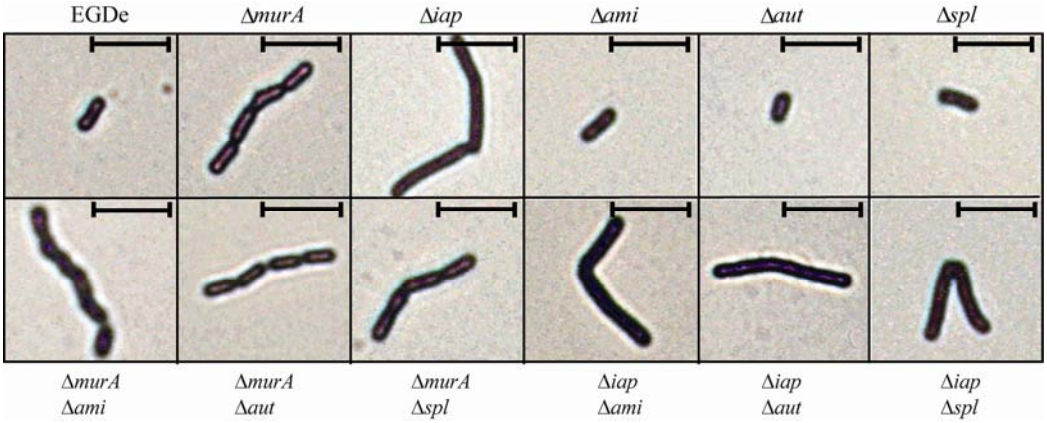


Figure 20: Morphology of single and double mutants in *L. monocytogenes* EGDe. Bacterial strains were grown to exponential phase (OD_{600} ~ 1.0), cells were Gram-stained and analyzed by light microscopy at 1,000 × original magnification. Scale bar 5 μm.

The deletion mutants Δ*ami*, Δ*aut* and Δ*spl* had a similar morphology to that of the wild type (Fig. 20). A combinatory double mutation with either *murA* or *iap* revealed a similar phenotype to the respective single mutants Δ*murA* or Δ*iap*.

3.1.5.3. Autolytic activity of deletion strains after Triton-X100 induction

To determine the role of the cell wall hydrolases and their involvement in cell destruction an autolysis assay was carried out with deletion mutants of EGDe. Autolysis of bacteria can be induced by various factors, the most basic being when cultures reach the maximal cellular density. Other factors include a change of pH or salt concentration as well was addition of antibiotics or chemicals can lead to autolysis in a bacterial culture. For our purpose, the detergent Triton-X 100 was used to cause autolysis in exponentially grown *Listeria* (24). Previous studies using Triton-X 100 demonstrated that the Δ*murA* and Δ*iap* deletion mutants have slightly reduced autolysis whereas the Δ*murA* Δ*iap* double mutant has highly reduced autolysis (93). Therefore those strains were used to compare autolytic activity with the newly created deletion mutants.The single mutant Δ*ami* displayed similar autolytic activity as the Δ*murA* and Δ*iap* deletion mutants whereas Δ*aut* had a more wild type-like phenotype (Fig. 21). Interestingly, the autolysis rate of the Δ*spl* mutant was increased, as evidenced by an optical density drop to less than 60% within the first hour (compared to ~ 80% for EGDe wild type). The bacterial density remained below wild type level throughout the seven hours of measurement (Fig. 21).

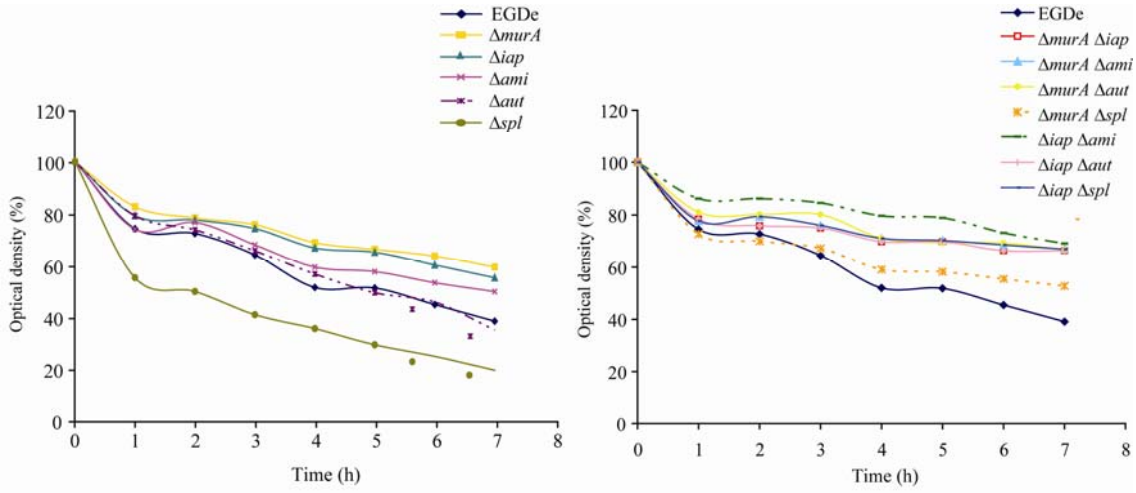


Figure 21: Triton-X 100 induced autolysis in *L. monocytogenes* wild type and deletion mutants. Bacteria were grown to exponential phase, chilled on ice and cells were resuspended in 50 mM glycine- 0.01% Triton X-100 buffer (pH 8.0). The culture was incubated at 37°C and the rate of autolysis was determined spectrophotometrically as a decrease in optical density at 620 nm.

Generally, the double mutants presented significantly lower autolysis rates than the single mutants. All combinatory deletions of either *murA* or *iap* with *ami* and *aut* resulted in similar autolysis rates as the previously reported strain Δ*murA* Δ*iap*. After seven hours of induction only ~30% of the cells were lysed with these strains whereas more than 50% of the wild type cells were disaggregated (Fig. 21). Only a light retardation in autolytic activity was found, however, with the double mutant lacking *murA* and *spl* (Fig. 21).

3.1.5.4. Tolerance to antibiotics and ethanol

L. monocytogenes is susceptible to a wide range of antibiotics (80). However, some strains are resistant to amdinocillin and methicillin and in general *L. monocytogenes* has a relatively high natural resistance to monobactams and some cephalosporins (44, 159). The treatment of choice for *L. monocytogenes* infections is either aminopenicillin or imipenem alone, or in combination with an aminoglycoside (71, 92). Bacterial resistance against various antibiotics was tested with the single and double mutants of *L. monocytogenes*. In several instances the susceptibility of various cell wall antibiotics varied. Alternations in the tolerance for vancomycin were found with the Δ*spl* single mutant and with the Δ*murA* Δ*spl* double mutant. Vancomycin is a glycopeptide-based antibiotic drug that prevents incorporation of *N*-acetyl-muramic acid (NAM)- and *N*-acetyl-glucosamine (NAG)-peptide subunits into the peptidoglycan matrix. It binds to the terminal D-alanyl-D-alanine moieties of the NAM/NAG peptides through formation of hydrogen bond interactions. Several other antibiotics belong to the group of cephalosporins that have the same mode of action as other β-lactam antibiotics (such as penicillins) and can be grouped into ‘generations’ by their antimicrobial properties. The first discovered cephalosporins were termed first generation and each newer generation had a more extended spectrum with significantly greater gram-negative antimicrobial properties and often with decreased activity against gram-positive organisms. Cefuroxim belongs to the second generation whereas Cefpodoxim, Cefotaxim and Ceftriaxon belong to the third generation. Generally, cephalosporins inhibit the formation of peptidoglycan cross-links in the bacterial cell wall. Their functional group, the β-lactam moiety, binds to the DD-transpeptidase that cross-links the peptidoglycan chains to form rigid cell walls. The double mutation of *murA* and *spl* exhibited increased sensitivity against the cephalosporins listed in Table 1. Also the double mutants Δ*iap* Δ*ami* and Δ*iap* Δ*aut* presented higher susceptibility against several cephalosporins compared to wild type or isogenic single mutants. *L. monocytogenes* is highly susceptible against penicillin but the *murA* and *iap*

mutants as well as Δ*iap* Δ*aut* and Δ*iap* Δ*spl* were even less tolerant to this β-lactam antibiotic.

Table 1: Antibiotic resistance of *L. monocytogenes* EGDe and deletion strains.

	EGDe	Δ*murA*	Δ*iap*	Δ*ami*	Δ*aut*	Δ*spl*	Δ*murA* Δ*ami*	Δ*murA* Δ*aut*	Δ*murA* Δ*spl*	Δ*iap* Δ*ami*	Δ*iap* Δ*aut*	Δ*iap* Δ*spl*
Vancomycin	-	-	-	-	-	+	-	-	+	-	-	-
Cefpodoxim	-	-	-	-	-	-	-	-	+	-	+	+
Cefuroxim	-	-	+	-	-	-	-	-	+	+	-	+
Cefotaxim	-	-	-	-	-	-	-	-	+	+	-	-
Ceftriaxon	-	+	+	-	-	-	+	-	+	-	-	-
Penicillin	-	(+)	(+)	-	-	-	-	-	-	-	(+)	(+)

(+) mild sensitivity; + sensitivity; ++ strong sensitivity; – no change

Furthermore, the deletion strains were tested for their stress tolerance by assessing the growth in BHI supplemented with 5% ethanol (Fig. 22). All single deletion strains are highly susceptible to ethanol and the strongest growth impairment was seen with the Δ*iap* and Δ*spl* mutants.

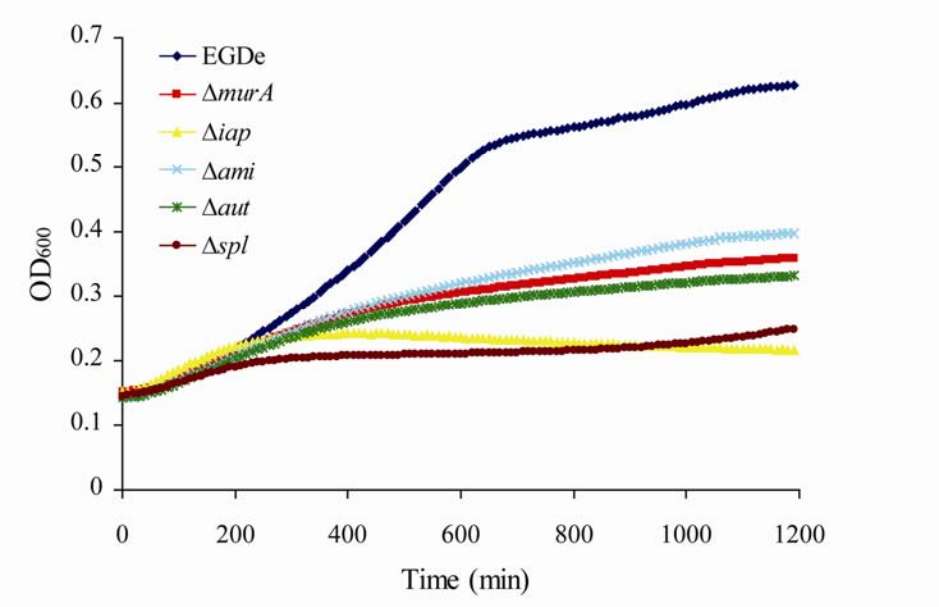


Figure 22: Growth of *L. monocytogenes* EGDe and deletion mutants under ethanol stress. Bacterial strains were shaken in BHI supplemented with 5% ethanol at 37°C and growth was determined by automated measuring of the optical density at 600 nm using the infinite series 200 plate reader (Tecan).

3.1.5.5. Lack of murein hydrolases impairs flagelllar motility

Autolysins are not only involved in cell division and cell stability but can also function in the incorporation of structural components into the cell wall. In *Salmonella typhimurium* the murein hydrolase FlgJ was shown to cleave the cell envelope at the site of

flagellar formation producing an *flgJ* mutant with decreased motility. Accordingly, the listerial deletion strains were tested for flagellar motility. Most *L. monocytogenes* strains are motile in soft agar plates at temperatures lower than 25°C (164). It has been shown that expression of flagellin FlaA encoding the major subunit of the flagellum is down-regulated at lower temperatures (40). The flagellar motility was determined by incubating step cultures of bacteria in 0.3% agar plates with BHI at 20°C for 4 days. The zone of growth diameter zone was measured for each strain and normalized to that of the wild type strain (set to 100%). A newly generated Δ*flaA* deletion strain was used as a negative control and displayed the lowest growth zone measuring only 12% that of EGDe (Fig. 23). The strains lacking *murA* and *iap* showed a strong deficiency in motility (< 40%) whereas the single mutations of *ami*, *aut* and *spl* had no or only a weak effect (>80%) on the swarming behavior of *L. monocytogenes* (Fig. 23). Surprisingly, the simultaneous deficiency of *murA* and *iap* did not decrease motility but improved it as compared to the single mutants (Fig. 23, ~70% vs. ~40%).

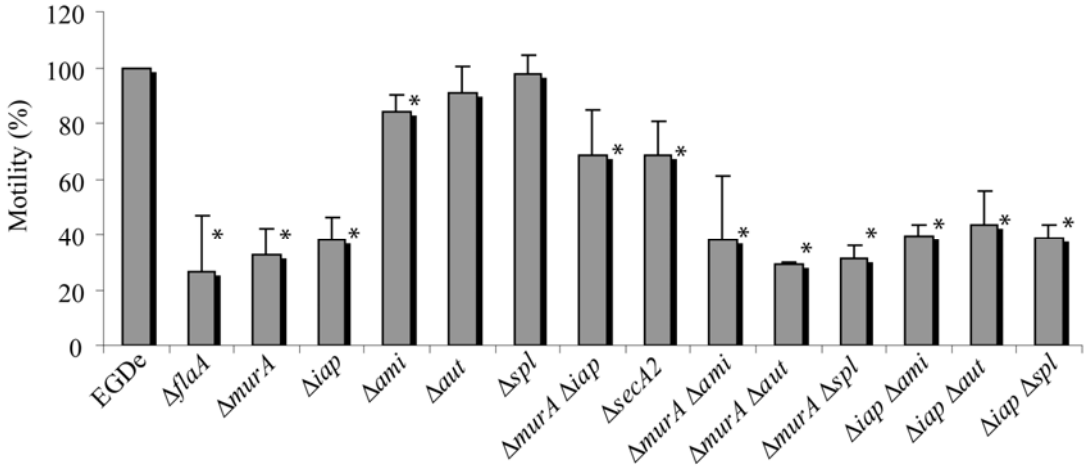


Figure 23: Flagellar motility of *L. monocytogenes* EGDe wild type and deletion mutants. Bacterial cultures were stabbed onto motility agar plates and incubated at 20°C for 2 to 4 days. * $p < 0.05$

3.1.5.6. Biofilm formation of rough variants and deletion mutants

A recent study showed that flagellar motility is critical for biofilm formation in *L. monocytogenes* (88). All deletion mutants lacking p60, namely Δ*iap*, Δ*iap* Δ*spl,* Δ*iap* Δ*aut*, Δ*iap* Δ*ami*, demonstrated decreased binding to plastic surfaces (Fig. 24) whereas the deletion strains Δ*spl*, Δ*aut* and Δ*mur* Δ*spl* had a wild type-like ability to form biofilms. A slight decrease in surface attachment was seen with the mutants Δ*murA* and Δ*ami*. The most dramatic decrease of colonization of plastic surfaces was found with the double mutants Δ*murA* Δ*iap* and Δ*murA* Δ*aut.*

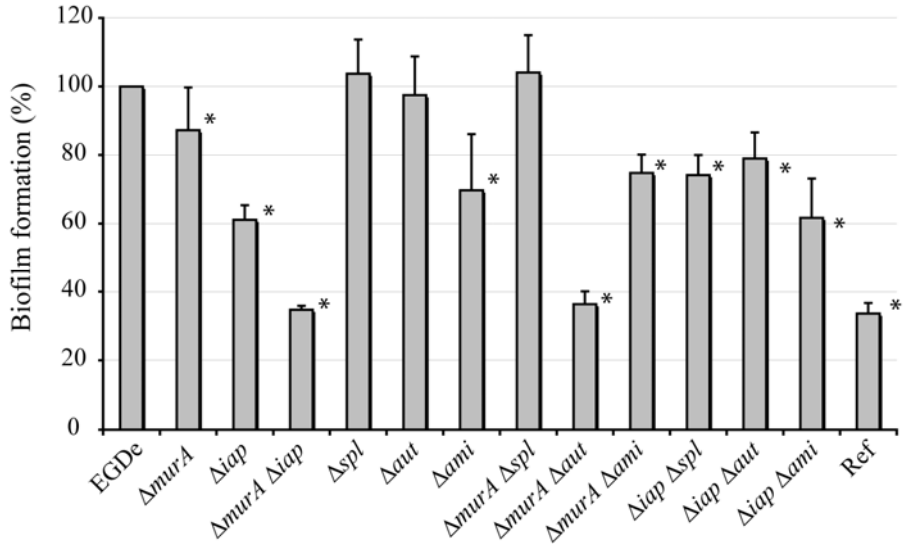


Figure 24: Biofilm of *L. monocytogenes* EGDe wild type and deletion mutants. Bacterial cultures in 96-well plates were grown for 72 h in LB at 20°C without shaking. The wells were washed in PBS and bacteria attached to the plastic surface were stained with crystal violet and absorption measured at 550 nm. * $p < 0.05$

3.1.5.7. Virulence studies

The influence of cell wall hydrolases on the virulence of *L. monocytogenes* has been studied previously for p60, MurA, Ami and Auto (20), (93), (103), (122). Deletion mutants for these proteins presented decreased adhesion, entry or survival properties (20, 93, 103, 122). A double deletion of *murA* and *iap* resulted in enhanced attenuation compared to their single mutants (93).

In the following section the newly created double deletion strains are compared to previously characterized mutants as well as Δ*spl* regarding their virulence in macrophages and epithelial cells. For invasion studies two cell lines, Caco2 and Hela, were used that carry different receptors on their surface and therefore require InlA or InlB activation for bacterial phagocytosis. After 2 h of infection the number of intracellular bacteria was determined by counting colony forming units (CFU) on BHI plates. Invasion of the Δ*iap* and Δ*murA* strains is significantly decreased as shown in Table 2. Simultaneous lack of both proteins increases the effect since the double mutant is even more impaired to enter both epithelial cell lines than the single mutants. The Δ*aut* deletion strain is slightly attenuated during infection of Hela cells but not with Caco2 cells. The Δ*ami* strain had only weak invasion defects and the Δ*spl* mutant had almost wild type-like invasion rates in both epithelial cell lines. Double mutation of *murA* and *ami* had similar effects as *murA* alone but the combined deletion of *murA* and *aut* led to a strongly decreased invasion rate of about 5%. Simultaneous deficiency of p45 with either MurA or p60 compensated the strong invasion defect of the single mutants

Δ*murA* or Δ*iap*, leading to increased invasion rates. Surprisingly, the double mutants with *spl* invaded Hela cells even better than the wild type EGDe. However, in Caco2 cells Δ*murA* Δ*spl* presented a decreased invasion rate of 40% compared to wild type. The most dramatic retardation of invasion in both epithelial cell lines was seen with the double mutants Δ*murA* Δ*iap*, Δ*murA* Δ*aut* and Δ*iap* Δ*ami*, with invasion rates of less than 8%.

Strains	Invasion Hela (%)	Invasion Caco2 (%)
EGDe	100	100
Δ*murA*	60* (± 0)	10 (± 0)
Δ*iap*	23* (± 23)	10 (± 0)
Δ*ami*	62* (± 17)	38* (± 12)
Δ*aut*	55* (± 18)	80 (± 16)
Δ*spl*	80 (± 26)	93 (± 7)
Δ*murA* Δ*iap*	4* (± 4)	1* (± 0)
Δ*murA* Δ*ami*	50 (± 14)	20 (± 9)
Δ*murA* Δ*aut*	6* (± 2)	5* (± 4)
Δ*murA* Δ*spl*	150* (± 4)	40 (± 27)
Δ*iap* Δ*ami*	4* (± 5)	7* (± 4)
Δ*iap* Δ*aut*	82 (± 4)	62* (± 15)
Δ*iap* Δ*spl*	352 (±223)	46* (± 7)

* $p < 0.05$

Table 2: Invasion rate of *L. monocytogenes* wild type EGDe and deletion mutants in the epithelial cell lines Hela and Caco2. Cells were infected for one hour and extracellular bacteria were killed by incubation with 50 µg/ml gentamicin for an additional hour. Bacteria were released from cells by lysis with 0.02% Triton and plated on BHI agar plates for quantification. The rate of invasion was determined as relative numbers of bacterial cells for the deletion strains with respect to the parent strain.

Once inside the cell *Listeria* are capable of spreading from one cell to the other as outlined earlier. These virulence properties can be tested by performing a plaque assay with L-929 fibroblast cells. Monolayers of L-929 cells are infected with exponentially growing bacteria and plaque formation occurs with strains forming actin tails and thus moving to adjacent cells. Previous studies with deletion mutants for MurA and p60 revealed smaller and fewer plaques for Δ*iap*, no plaque formation with Δ*murA* Δ*iap* and no difference between wild type and Δ*murA* (93). The Δ*aut* and Δ*spl* deletion strains, used for this study, acted in a similar manner as Δ*murA* since infection of L-929 cells resulted in only slightly reduced plaque size and similar numbers (Fig. 25A). Surprisingly, the double mutation of *murA* and *spl* resulted in a strong infection deficiency with 44% smaller and 57% less plaques as compared to EGDe wild type (Fig. 25A). Deletion of the *ami* gene also caused significant changes leading to reduced plaque size (61%) and decreased quantity of plaques (64%) (Fig. 25A). Even more interesting was the effect seen with the double mutants Δ*murA* Δ*ami*, Δ*murA* Δ*aut* and Δ*iap* Δ*ami*. These strains appear unable to infect the fibroblast cell line L-929 as there was no plaque formation at all (Fig. 25A).

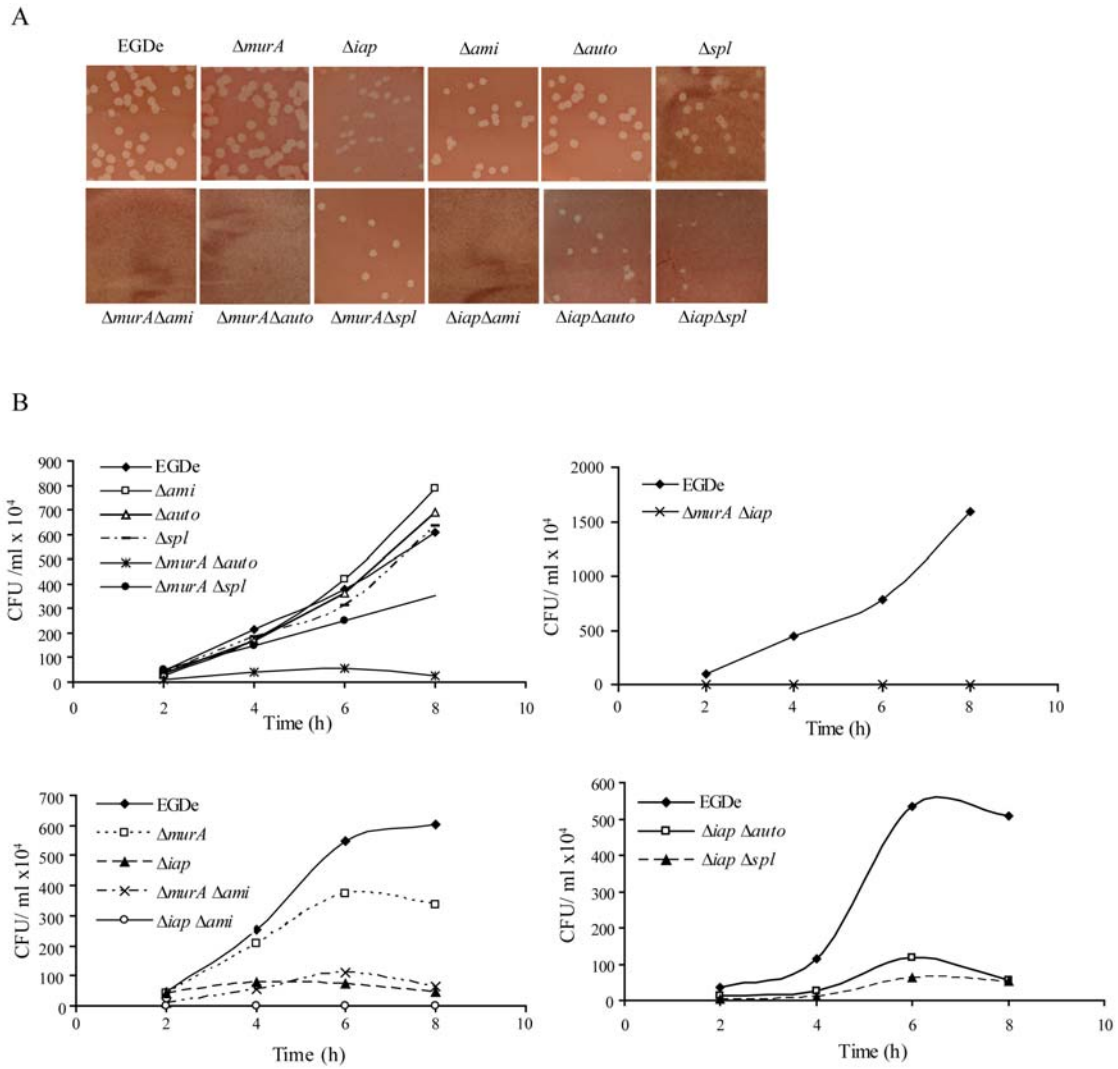


Figure 25: *In vitro* infection of fibroblasts and macrophages with EGDe wild type and deletion mutants. **(A)** Cell to cell spreading of bacteria assayed by plaque formation in L-929 fibroblasts. **(B)** Intracellular replication of bacteria within P388D1 macrophages analyzed at 2, 4, 6 and 8 h.

Finally, intracellular survival and propagation of the deletion strains were assessed using the macrophage cell line P388D1. These cells do not require activation of phagocytosis for bacterial uptake. They can be used to study intracellular survival and propagation independent of the adhesion and invasion properties of the bacterial strain. The intracellular growth of the previously described deletion strain Δ*murA* was slightly decelerated and that of Δ*iap* was dramatically decreased (Fig. 25B). The effect is even more pronounced with a double mutation of both genes leading to complete failure to grow intracellularly (Fig. 25B). However, individual deletion of *ami*, *aut* or *spl* does not affect intracellular survival and replication as these strains showed similar growth kinetics as the wild type EGDe. Double

deletion of *murA* and *spl* leads to similar growth kinetics as presented with the Δ*murA* deletion strain. Similarly, double deletion of *iap* and either *spl* or *aut* showed no difference in growth behavior versus the *iap* single mutant. In contrast, no growth could be observed in macrophages infected with the Δ*iap* Δ*ami* mutant. *S*imultaneous lack of *murA* and *ami* or *murA* and *auto* also results in strong inhibition of intracellular propagation (Fig. 25B).

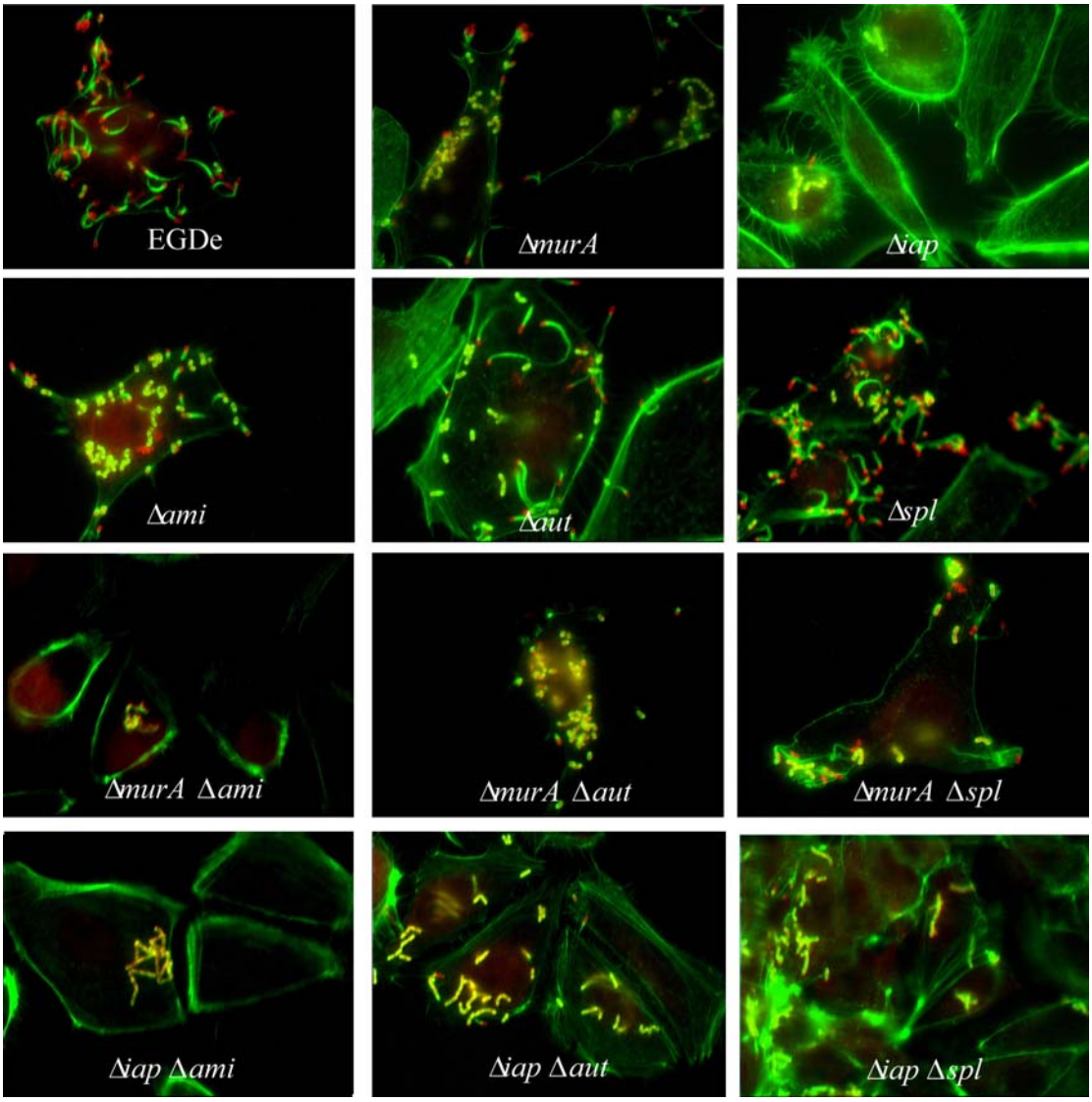


Figure 26: PTK2 epithelial cells infected with EGDe wild type and deletion mutants analyzed by immunofluorescence microscopy. Cells were fixed with 3.7% formaldehyde in PBS after 2 h infection. Actin filaments of eukaryotic cells were stained with oregon green phalloidin (green) and bacteria were visualized via anti-ActA antibody (red).

To obtain a better idea of the defects caused by the various deletions during infection, infected cells were and analyzed by immunofluorescence microscopy using anti-ActA

antibody for detection of bacteria and phalloidin for detection of actin filaments in eukaryotic cells. The PTK2 epithelial cell line was used for this study (Fig. 26). The single deletion mutants Δ*aut* and Δ*spl* present virulence properties similar to the parental strain EGDe (Fig. 20). Bacteria have long actin tails (green) and efficiently spread out of the cell. We observed, as reported previously (122), that the Δ*iap* deletion strain is deficient in polarizing actin and therefore forms no actin tails. The Δ*murA* and Δ*ami* strains display retardation in forming actin tails whereas the effect is less pronounced than with Δ*iap*. All double mutants were strongly impaired in building actin tails and spreading to neighboring cells.

3.1.5.8. Summary of effects seen by simultaneous deletion of several hydrolases

The various phenotypes observed with single or double deletion of murein hydrolases in *L. monocytogenes* are summarized in Table 3. In general the pairwise combinatory deletion of the five murein hydrolases MurA, p60, Ami, Auto and p45 leads to cumulative deficiencies as observed with the single deletion mutants. This is demonstrated with the double mutants Δ*murA* Δ*ami* and Δ*iap* Δ*ami* that show severely impaired virulence to a higher extent than seen with individual single mutants. However, double mutation of *spl* with either *murA* or *iap* shows synergistic effects in virulence, for example enhanced biofilm formation and increased invasion of epithelial cells compared to the single deletion mutants Δ*murA* and Δ*iap*.

Table 3: Effects of single and double deletion of the genes *murA*, *iap*, *ami*, *aut* and *spl* on morphology, biological fitness and virulence of *L. monocytogenes*.

STRAINS	EGDe	Δ*murA*	Δ*iap*	Δ*ami*	Δ*aut*	Δ*spl*	Δ*murA* Δ*ami*	Δ*murA* Δ*aut*	Δ*murA* Δ*spl*	Δ*iap* Δ*ami*	Δ*iap* Δ*aut*	Δ*iap* Δ*spl*	Δ*murA* Δ*iap*
Morphology													
Colony morphology	smooth	smooth	smooth	smooth	smooth	smooth	smooth	smooth	smooth	smooth	smooth	smooth	rough
Cell morphology	single cells	chains	chains	single cells	single cells	single cells	chains	chains	chains	chains	chains	chains	long chains
Biological fitness													
Autolyse	+	-	-	-	+	+ +	- -	- -	-	- -	- -	- -	- -
Ethanol	+	-	- -	-	-	- -							
Motility	+	- -	- -	-	+	+	- -	- -	- -	- -	- -	- -	-
Biofilms	+	-	- -	+	+	+	-	- -	+	- -	-	-	- -
Virulence													
Invasion into Hela	100	60	23	62	55	80	50	6	150	4	82	352	4
Invasion into Caco	100	10	10	38	80	93	20	5	40	7	62	46	1
Growth inP388D1	+	-	- -	+	+	+	- -	- -	-	- -	- -	- -	- -
Actin tails by IF	long	short	none	short	long	long	none	none	none	none	none	none	none
Cell to cell spread	+	+	-	-	+	+	- -	- -	-	- -	-	-	- -

+ wild type; ++ positive effect; - defect; -- strong defect

A striking observation is the highly increased invasion of the Δ*murA* Δ*spl* and Δ*iap* Δ*spl* strains into Hela cells that significantly exceeds the virulence properties of the EGDe wild type. Double mutation of *aut* with *murA* has effects that have not been observed with the single mutants Δ*auto* or Δ*murA*. The Δ*murA* Δ*aut* double deletion mutant is highly attenuated in virulence with strongly decreased invasion and propagation properties and the inability to spread from cell to cell in fibroblasts. Simultaneous deficiency of p60 and Auto gives rise to a phenotype similar to Δ*iap*. However, none of the combinatory deletions with *ami*, *aut* or *spl* caused a rough phenotype, which is found with double deletion of *murA* and *iap*.

3.1.6. Analysis of additional putative murein hydrolases in *L. monocytogenes*

3.1.6.1. Generation and characterization of deletion mutants

Data presented in this study illuminate the significance of autolysins for various cellular processes in *L. monocytogenes*. We therefore attempted to identify the role of other proteins in this microorganism that share common functional domains with murein hydrolases. For this purpose, genes encoding the putative autolysins listed in Figure 11B were deleted in strain *L. monocytogenes* EGDe. Successful deletion was verified by sequencing the region of interest for each mutated strain. Mutant strains were compared with the wild type in a series of experiments to determine the function of the deleted genes.

3.1.6.2. Morphology and growth

As a start the cell morphology of the newly generated deletion strains was examined. Light microscopy analysis did not reveal any differences in cell shape compared to the wild type EGDe. All strains had separated, rod shaped cells with smooth colony morphology when grown on BHI agar plates (data not shown). The deletion mutants also presented wild type-like growth properties when cultivated at 37°C in rich liquid media such as BHI broth. Cultivation at lower temperatures (15°C, 4°C) on BHI agar plates indicated no phenotypic changes of the mutated strains.

3.1.6.3. Protein and lytic profile

The deletion strains were further investigated by analyzing their protein profile and lytic activity with cell wall material. Protein profiles were assessed from culture supernatant, cell wall, cytoplasm and membrane via separation by SDS-PAGE and staining with Coomassie blue. The autolytic enzyme profile of the mutated strains was analyzed on a SDS

polyacrylamide gel containing cell wall of *L. monocytogenes*, *Micrococcus lysodeikticus* and *B. subtilis*. The proteins were separated by electrophoresis and renatured by incubation in a 0.01% Triton-X 100 buffer in 25 mM Tris-HCl (pH 7) with lytic bands appearing as clear zones in the opaque gel. The three substrates used for this assay, *Listeria*, *Micrococcus* and *Bacillus*, demonstrate that the murein hydrolases of *L. monocytogenes* EGDe have diverse ligand specificities (Fig. 27). However, none of the deletion mutants showed differences in lytic activity compared to the wild type.

Western blots were carried out to determine the influence of the putative cell wall hydrolases on the expression of the characterized autolysins MurA and p60 as well as listerial virulence factors such as InternalinA and B, Listeriolysin and ActA. All deletion mutants presented wild type like expression of virulence- associated proteins tested by immunoblot analysis.

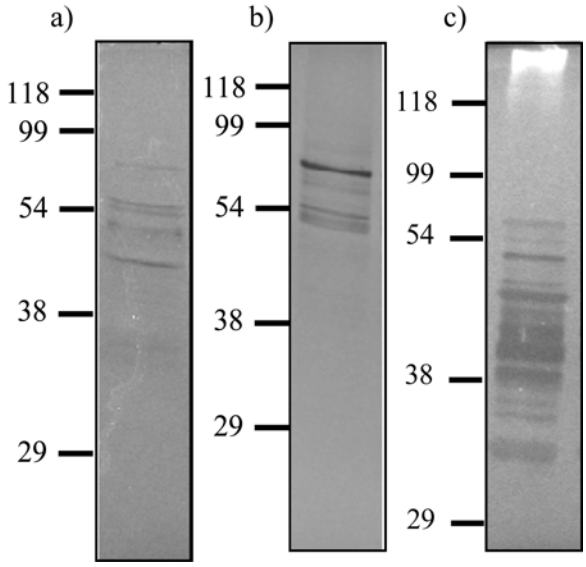


Figure 27: Lytic profiles of SDS- fractionated proteins of *L. monocytogenes* EGDe with: (a) *Bacillus*, (b) *Micrococcus* and (c) *Listeria* substrate.

3.1.6.4. Growth under stress conditions

Since murein hydrolases are involved in resistance against various antibiotics, we tested the growth of the newly created deletion strains of *L. monocytogenes* and the parental strain on BHI agar plates containing small discs having diverse antibiotics. There was no significant difference between the resistance profile of wild type compared to the deletion mutants. Only one strain, Δ*1104*, showed increased sensitivity against two cephalosporins, Cefuroxim and Ceftazidim. Cefuroxim belongs to the second and Ceftazidim to the third generation of cephalosporins, β-lactam antibiotics that inhibit cross-linking of the bacterial murein layer.

The strains were also tested for growth in BHI with addition of 5% ethanol to determine the influence of stress factors on the modification of the cell wall. Very strong growth deficiencies under ethanol stress conditions were observed for strains having a deletion of Lmo2522, a glucosaminidase (Δ*1215* or Δ*2591*), a muraminidase (Δ*0717*) and a peptidase (Δ*0394* or *lmo1104*) (Fig. 28). Also, the lack of the putative glucosaminidases Lmo1216 and Lmo2203, the amidases Lmo0129 as well as the LysM proteins Lmo1303 and Lmo1941 led to a less prominent but still significant attenuation of growth (Fig. 28). Only the Δ*1521* mutant strain showed wild type-like growth under ethanol stress conditions. The proteins lacking in these strains might therefore be important for maintenance of the cell shape during stress.

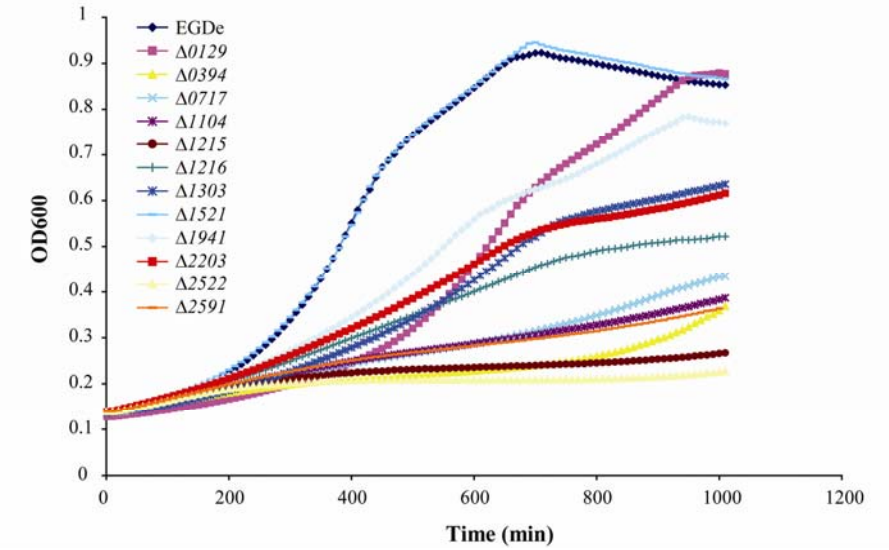


Figure 28: Bacterial growth under ethanol stress of deletion mutants lacking putative murein hydrolases. Bacterial strains were shaken at 37°C in BHI supplemented with 5% ethanol and growth was determined by automated measuring of the optical density at 600 nm using the infinite series 200 plate reader (Tecan).

3.1.6.5. Flagellar motility and biofilm formation

Flagellar motility was tested for the newly described deletion mutants of *L. monocytogenes* EGDe via a cell spreading-assay. None of the strains displayed inhibitory effects as presented previously with the Δ*murA* and Δ*iap* mutants. Nevertheless, a few strains (Δ*0717*, Δ*1521* and Δ*1941*) were ~20% less motile than the wild type and the other deletion mutants (Fig. 29).

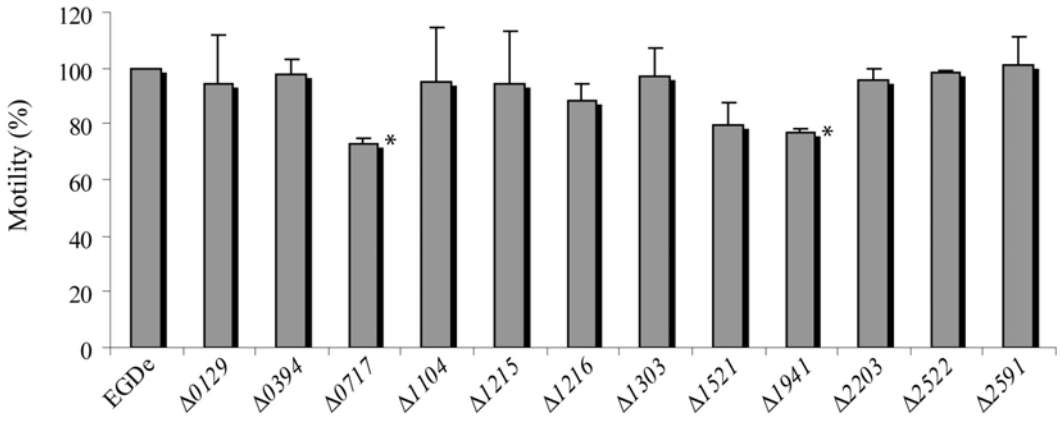


Figure 29: Motility of *L. monocytogenes* EGDe wild type and deletion strains. * $P < 0.05$

Additionaly, all strains were analyzed for biofilm formation on plastic and displayed a similar profiles to wild type EGDe.

3.1.6.6. Virulence studies

The characterization of growth and appearance of the deletion mutants was followed up by studies on the interaction of the bacteria and host cells. Various assays with epithelial cells, fibroblasts and macrophages were performed as previously described to investigate the virulence of the mutant strains. There was no significant reduction of invasion seen after infection of Caco2 cells. Instead, the Δ*2591* deletion strain showed a 50% higher invasion rate that the EGDe wild type. With infection of Hela cells a few strains showed less than 80% invasion, among those Δ*1104*, Δ*1216*, Δ*1303* and Δ*2203*. However, only the data obtained with infection of Δ*1104* showed a p-value of less than 0.05 and can therefore be considered significant.

Strains	Invasion Hela (%)	Invasion Caco2 (%)
EGDe	100	100
Δ0129	92 (± 1)	108 (±13)
Δ0394	123 (±29)	96 (±31)
Δ0717	84 (±18)	117 (±10)
Δ1104	*76 (± 5)	112 (±24)
Δ1215	97 (±49)	86 (±19)
Δ1216	63 (±17)	89 (±20)
Δ1303	79 (±21)	95 (±18)
Δ1521	164 (±48)	96 (±30)
Δ1941	109 (±43)	89 (±31)
Δ2203	75 (±13)	93 (±51)
Δ2522	124 (±41)	90 (± 9)
Δ2591	94 (±28)	147 (±48)

* $p < 0.05$

Table 4: Invasion rate of *L. monocytogenes* wild type EGDe and deletion mutants in the epithelial cell lines Hela and Caco2
Infection was performed as described in Materials and Methods and the invasion rate was defined in comparison to the wild type EGDe.

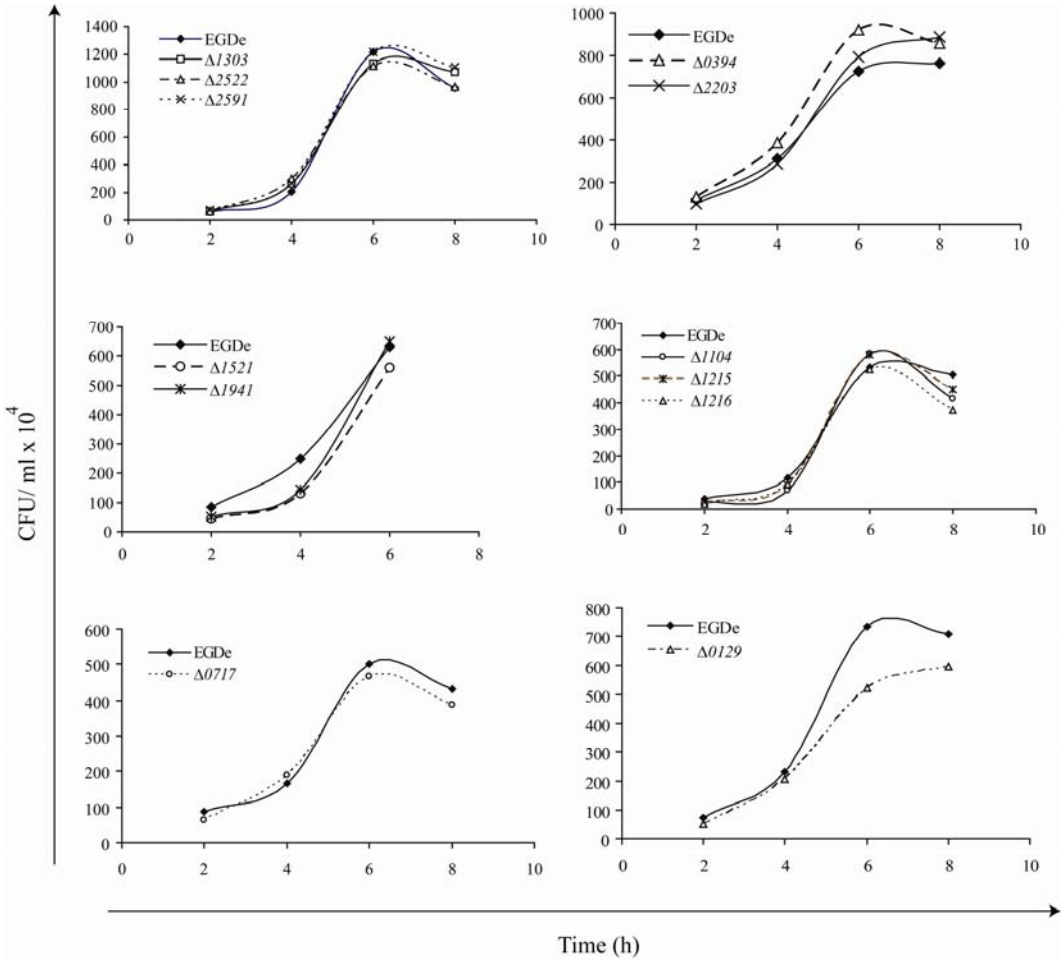


Figure 30: Growth of EGDe and deletion mutants in P388D1 macrophages. Infection was performed as described in Materials and Methods.

In general, the mutants showed no significant differences in intracellular replication in the macrophage cell line P388D1 (Fig. 30) or in spreading in L-929 monolayers (data not shown) compared to the EGDe wild type. Only the Δ*0129* deletion strain displayed a slight decrease in growth 6 and 8 hours post infection. Immunofluorescence microscopy of the epithelial cell line PTK2 did not reveal any changes in infection behavior observed with the deletion mutants compared to their wild type (Fig. 31).

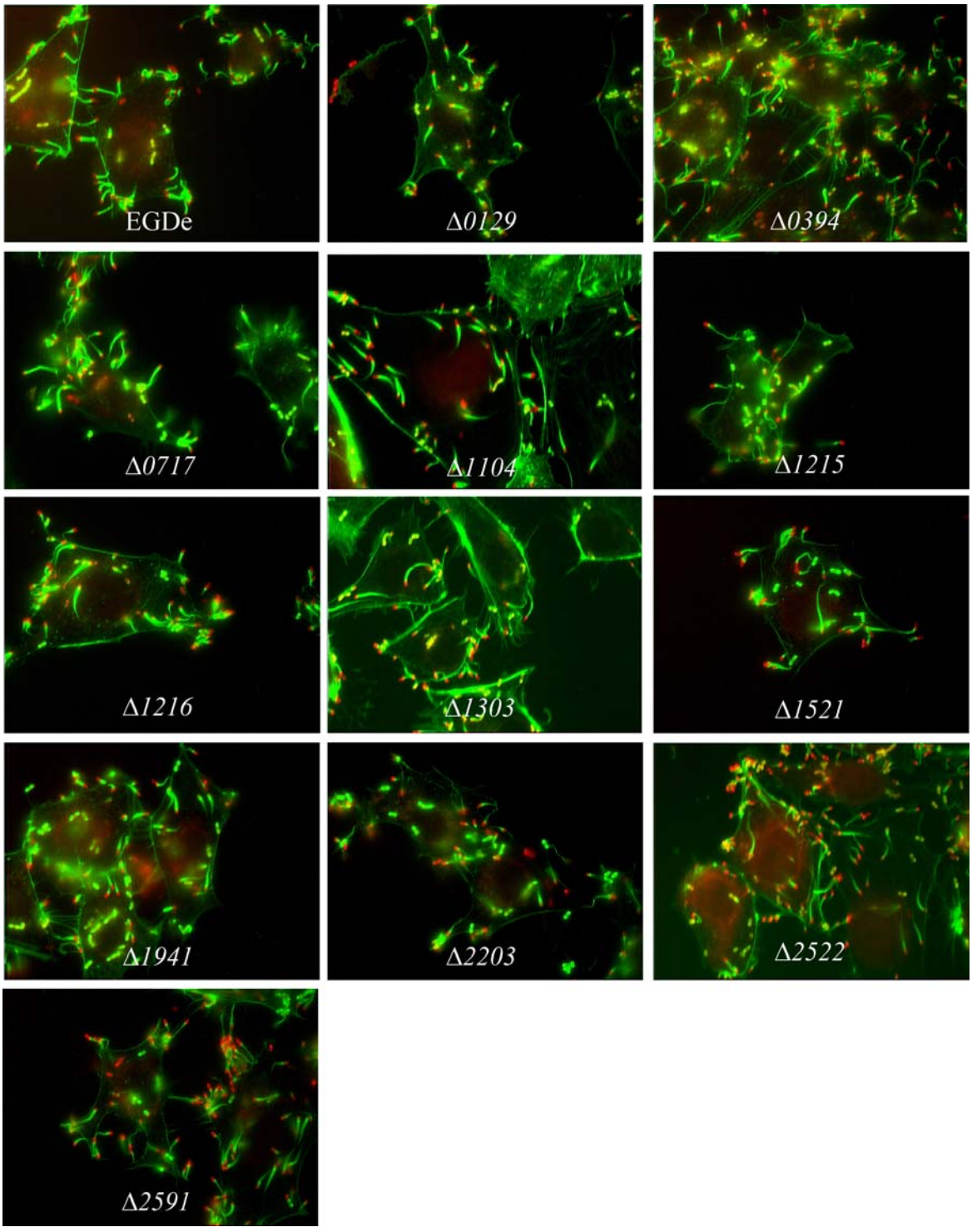


Figure 31: Immunofluorescence microscopy of *L. monocytogenes* wild type and deletion strains during infection. The epithelial cell line PTK2 was infected with bacteria for 2 hours, followed by staining of cellular actin with Oregon-green (green) and of bacteria with monoclonal antibody directed against ActA (red).

3.2. Effects of cationic antimicrobial peptides (CAMPs) on *L. monocytogenes*

3.2.1. Background

L. monocytogenes contains two different polyanionic polymers decorating the cell wall: the teichoic acids (TAs) that are covalently bound to the peptidoglycan and the lipoteichoic acids (LTAs) that are docked in the cellular membrane by glycolipid anchor. The composition of lipoteichoic acids, in particular their D-alanine content, has been shown to influence immune response and survival in a murine colitis model of lactic acid bacteria (55). The D-alanine residues are formed from L-alanine by an alanine racemase and coupled to the LTA by the coordinated activity of four proteins DltA-D encoded by the *dlt* operon (Fig. 32A). The DltA protein activates D-alanine and ligates the activated ester to the 4'-phospho-pantetheine prosthetic group of the D-alanyl carrier protein DltC. DltB is a transport protein involved in efflux of activated D-alanine to the site of acylation. DltD is a membrane-associated protein that may have multifunctional activities (hydrolysis of mischarged DltC, facilitation of D-alanine ligation to DltC and D-alanylation of LTAs) (1, 100). A schematic overview of this process is presented in Figure 32B.

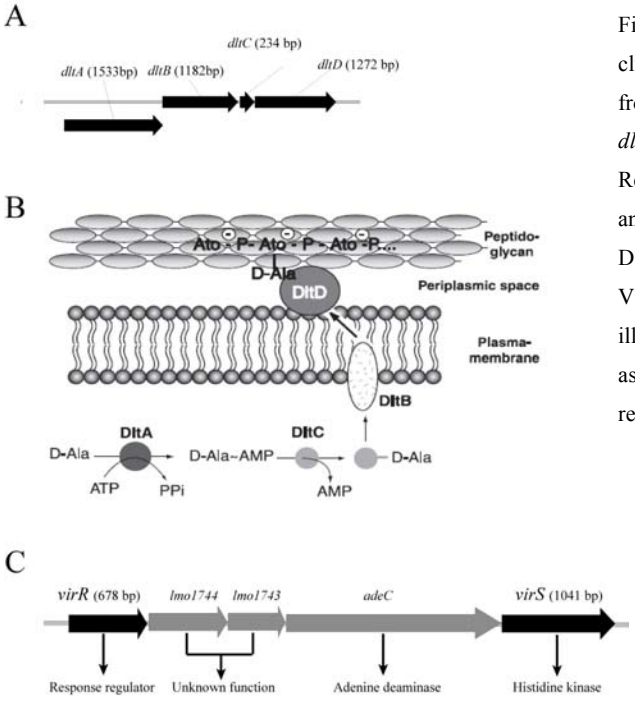


Figure 32: D-Ala biosynthesis gene clusters and VirR regulon gene cluster from *L. monocytogenes* (A) Genes of the *dlt* operon are depicted as arrows. (B) Reaction catalyzed by the proteins DltA and DltC. Ato, alditol; P, phosphate; DltA-D, proteins. (C) Genes of the VirR/VirS two component system are illustrated as black arrow, genes between as grey arrows. (Adapted from references (95, 100)).

The esterification of teichoic acids with D-alanine has the consequence that positively charged amino groups are introduced into the cell envelope thus decreasing the net negative

charge on the bacterial surface. This change in negative surface charge has been implicated as a resistance mechanism of gram-positive bacteria against cationic antimicrobial peptides with membrane-damaging activity (23). A deficiency in D-alanine incorporation into LTAs leads to increased electronegativity of the cell surface thereby allowing cationic compounds to bind more efficiently (5). Cationic antimicrobial peptides (CAMPs) are important for human defenses in protecting skin and epithelia against invading microbes and in assisting neutrophiles and platelets (116). The reduction of the D-alanyl content of the cell wall is also thought to influence the autolysis mechanism. Whereas absence of D-alanine from the polymers has been reported to cause increased autolysis in *B. subtilis* (165), studies with a *dltA*-deficient mutant of *S. aureus* showed the opposite effect; namely, a reduced rate of autolysis (118).

Recently, a new regulator for virulence, VirR, in *L. monocytogenes* was identified via a deletion mutant displaying attenuation during infection (95). This regulatory protein was shown to control a series of genes among those the *dltABCD* operon and *mprF* of *L. monocytogenes*. The protein encoded by *mprF* modifies membrane phosphatidyl glycerol with L-lysine and is involved in resistance to human defensins (117, 152). For *Salmonella typhimurium* a sensor kinase has been shown to be activated by CAMPs, leading to changes in genes expression and enabling the bacterium to respond to the host immune system. It was proposed in our study that the listerial VirR system also regulates resistance to cationic peptides since the regulated genes, *mprF* and the *dlt* operon, have an influence on the overall charge of the bacterial surface. The VirR regulator is part of a two component system together with the sensor kinase VirS (Fig. 32C)

3.2.2. Characterization of mutants defective in D-Ala incorporation into lipoteichoic acid (LTA)

3.2.2.1. Construction and characterization of deletion mutants Δ*dltB*, Δ*dltC*, Δ*virR*, Δ*virS* and Δ*dal*

It was of interest to study the interplay of the *virR* regulatory system and the *dlt* operon for survival and virulence of *L. monocytogenes*. Previous studies with a Δ*dltA* deletion mutant in *L. monocytogenes* exhibited increased susceptibility to cationic peptides (such as Nisin, Colistin and Polymyxin B) and severely impaired infection properties in a mouse model and *in vitro* with various cell lines (1). The authors concluded that D-alanylation of the LTAs contributes to the virulence of the intracellular pathogen *L.*

monocytogenes. A Δ*virR* deletion mutant was shown to be more strongly impaired in virulence leading to decreased bacterial loads in infected mice and diminished entry of Caco2 cells. In this work, deletion mutants were constructed for genes of the *dlt* operon as well as *virR* and *virS* genes. Specifically, the *dlt* operon of *L. monocytogenes* was characterized by constructing D-Ala deficient mutants lacking *dltB* or *dltC*. Attempts to generate deletion strains for *dltA* and *dltD* were not successful even though Δ*dltA* mutants have been reported (1, 95).

In *Listeria monocytogenes*, the conversion of L-alanine to D-alanine is performed by the alanine racemase encoded by *dal* (153). The bacterium also has an alternative pathway to generate D-alanine through the activity of D-amino acid aminotransferase (*dat*). Simultaneous mutation of the genes *dal* and *dat* leads to a dependence on exogenous D-alanine for proper growth (153). To understand the importance of the alanine racemase for D-alanine assembly into LTAs, a deletion mutant of the *dal* gene was also generated in this work.

The phenotypes of the Δ*dltB*, Δ*dltC*, Δ*virR*, Δ*virS* and Δ*dal* mutants were examined with various assays for morphology, growth, autolysis and motility. None of the strains showed significant differences compared to the wild type *L. monocytogenes* EGDe (data not shown). However, considerable changes were found when the mutants were tested for antibiotic resistance. An antibiogram revealed increased sensitivity of the Δ*dal* mutant to a few cephalosporins of different generations (Cefuroxim (2nd), Cefotaxim (3rd), Ceftriaxon (3rd) and Cefepim (4th)). A much stronger growth defect was seen with antibiotics that act as cationic peptides, such as Polymyxin B, Gallidermin and Colistin. The growth of deletion strains Δ*dltB*, Δ*dltC*, Δ*virR* and Δ*virS* was completely inhibited as shown in Figure 33A-C. In contrast, the Δ*dal* strain had only a slight growth defect in the presence of cationic peptides.

The cationic peptides initially tested so far are of bacterial origin and have no relevance for *L. monocytogenes* during host infection. Therefore, we also tested a cationic peptide produced by eukaryotic host cells. The LL-37/human cationic antimicrobial protein 18 (LL37) consists of 37 amino acids and is synthesized by macrophages, neutrophiles, and epithelial cells (providing antimicrobial protection to skin and the lining of the urinary tract). LL37 is a member of the cathelicidins and plays a major part in the innate immune response in the respiratory epithelium (156). Growth in the presence of LL37 (kindly provided by R. Bals (Institute of Pneumology, Marburg University) was assessed for the Δ*virR* and Δ*virS* mutants. All strains reached stationary phase after almost 8 hours when cultured in BHI broth

(Fig. 33D). However, growth of the Δ*virR* or Δ*virS* deletion strains was completely arrested in broth supplemented with the antimicrobial peptide (Fig. 33D, white columns). Proliferation of the wild type EGDe, though not inhibited, was slightly delayed during cultivation with 50 µg/ml of the LL37 peptide.

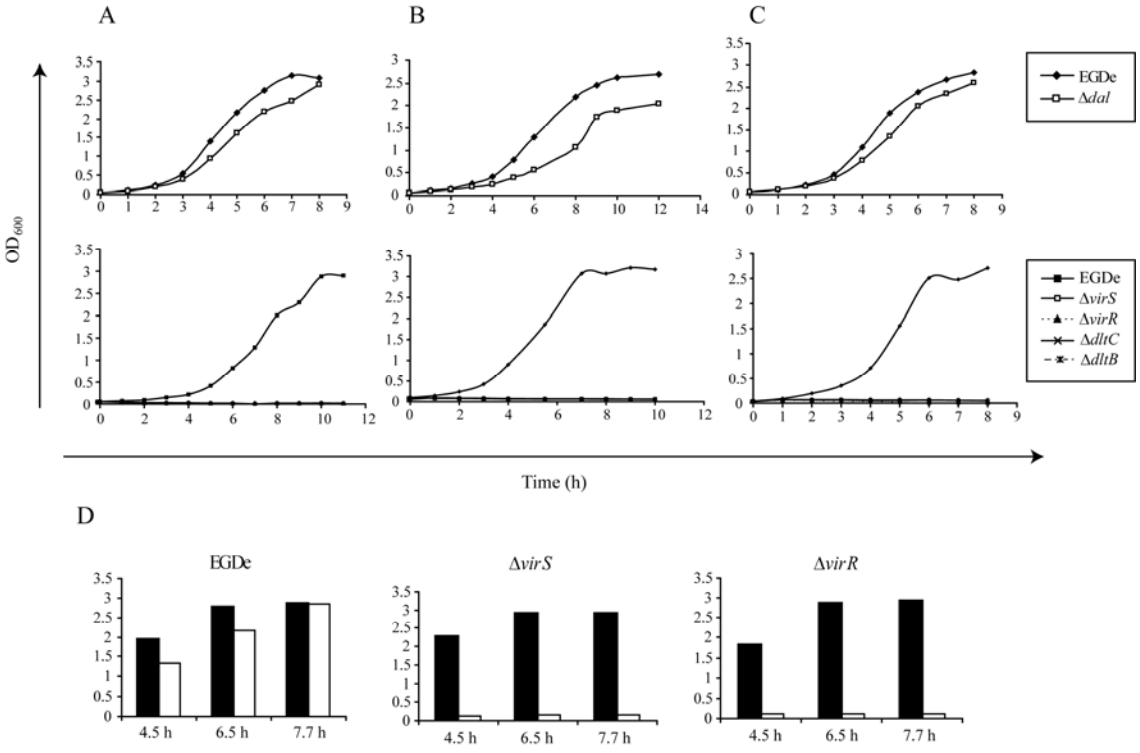


Figure 33: Growth of *L. monocytogenes* EGDe wild type and of deletion mutants Δ*dal*, Δ*dltB*, Δ*dltC*, Δ*virR* and Δ*virS* in the presence of cationic peptides. Bacteria were cultured in BHI broth supplemented with (A) 35 µg/ml Colistin, (B) 20 µg/ml Polymyxin B or (C) 0.5 µg/ml Gallidermin and the OD_{600} was measured hourly. The sensitivity to human CAMP LL37/ /hCAP-18 was tested for EGDe wild type and Δ*virR* and Δ*virS* deletion mutants (D). Growth of *L. monocytogenes* EGDe in BHI supplemented with water (black columns) or 50 µg/ml LL37 /hCAP-18 (white columns) was monitored by measuring the optical density at 600 nm.

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The importance of the *dlt*-operon and the VirR/S system for bacterial resistance against CAMPs was also analyzed by quantitative real time-PCR. The EGDe wild type was cultured in BHI supplemented with Polymyxin B or Colistin. Cells were harvested at exponential phase and RNA was isolated and reverse transcribed. The expression pattern of *dltA, mprF*, *virR* and *virS* was tested by quantitative real time PCR and compared to that of bacteria cultured in BHI only. Whereas the *dltA* and *mprF* genes were found to be up-

regulated during growth in the presence of Colistin or Polymyxin B (Fig. 34), genes of the VirR/VirS regulon showed no significant changes.

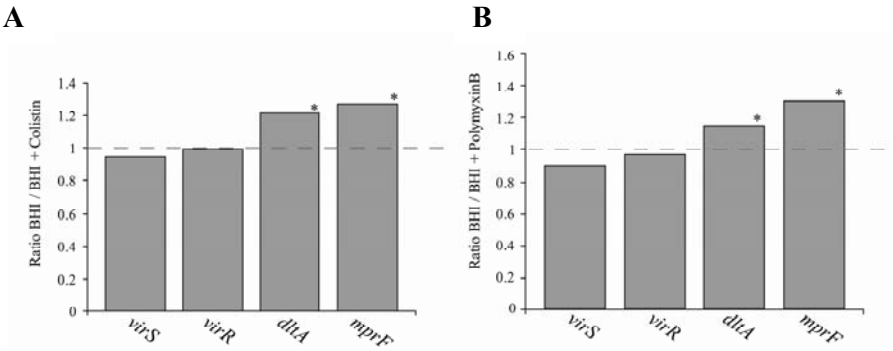


Figure 34: Quantitative real-time PCR of *dltA*, *mprF*, *virR* and *virS* inEGDe in the presence of cationic peptides. *L. monocytogenes* EGDe was grown fo exponential phase in BHI supplemented with **(A)** 40 µg/ml Colistin or **(B)** 30 µg/ml Polymyxin B and cells were harvested for RNA isolation and subsequent RT real time PCR. The genes *dltA*, *mprF*, *virS* and *virR* were quantified according to Chatterjee (2006) (28) by using standard curves obtained from genomic DNA. The values were normalized to bacteria grown in BHI without addition of antibiotics.* $p < 0.05$.

Bacterial sensitivity to ethanol stress was also tested. Whereas the deletion mutants for *virR*, *virS*, *dltB* and *dltC* exhibited growth similar to the EGDe wild type, the *dal* deletion mutant was shown to be more vulernable against ethanol (Fig. 35).

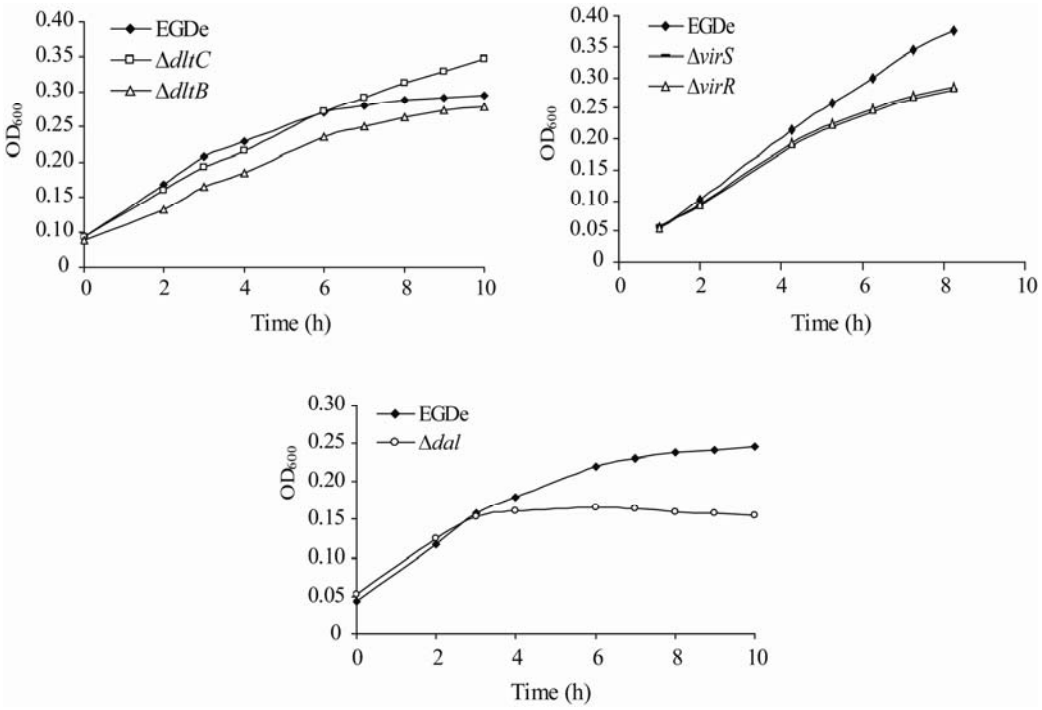


Figure 35: Sensitivity of deletion mutants Δ*dal,* Δ*dltB,* Δ*dltC,* Δ*virS* and Δ*virR* to ethanol stress.

3.2.2.2. Virulence properties of the mutant strains

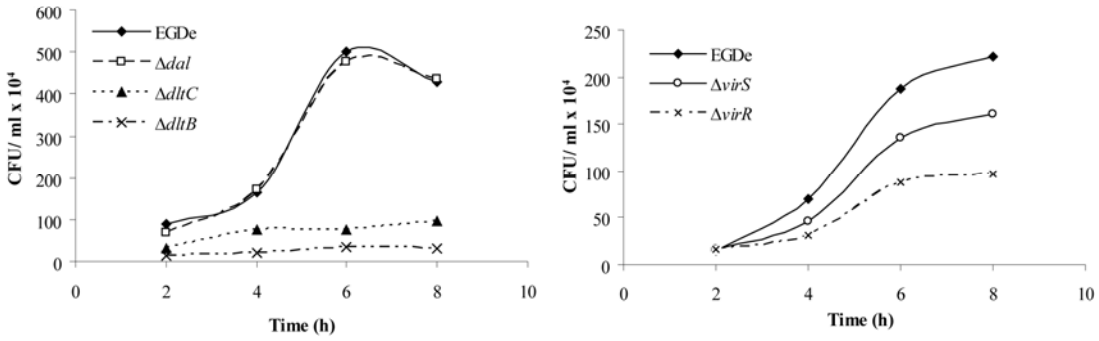
We were also interested in the virulence properties of the newly created deletion strains of *L. monocytogenes*. Invasion was tested as previously described with human epithelial cells. Surprisingly, the deletion strains displayed a large difference in their invasion of Caco2 versus Hela cells. Whereas the invasion rate of Hela cells was almost wild type level (with the exception of Δ*dltB* with 38 %), there was a strong invasive defect with the Caco2 cell line (Fig. 36A). The Δ*dlt* and Δ*vir* deletion mutants had invasion rates of only 0.4 to 3%.

A

Strains	Invasion Hela (%)	Invasion Caco2 (%)
EGDe	100	100
Δ*dal*	119 (± 13)	63* (±21)
Δ*dltC*	87 (± 25)	0.4* (± 0)
Δ*dltB*	38* (± 13)	2* (± 2)
Δ*virS*	112 (± 34)	2* (± 2)
Δ*virR*	110 (± 34)	3* (± 3)

* $p < 0.05$

B



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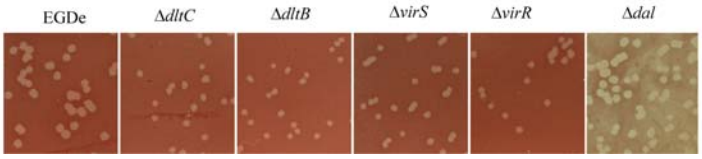

Figure 36: *In vitro* virulence assays with *L. monocytogenes* EGDe and deletion mutants Δ*dltC*, Δ*dltB*, Δ*virR*, Δ*virS* and Δ*dal*. **(A)** Invasion into the human epithelial cell lines Hela and Caco2, **(B)** intracellular replication in P388D1 macrophages and **(C)** intracellular spreading in L-929 fibroblasts.

Our next approach to analyze the virulence of the CAMP sensitive strains was to test intracellular survival in the macrophage cell line P388D1. Intracellular replication was found to be dramatically decreased with all deletion strains (Fig. 36B). The ability of the deletion strains to spread from one cell to another was assayed by infection of fibroblast monolayers

and monitoring plaque formation after incubation for several days. All mutant strains were capable of successfully infecting the L-929 cell line however plaque sizes of the Δ*dltB/C* and Δ*virR/S* strains were about 50% reduced compared to the wild type (Fig. 36C). Significant inabilit of the Δ*virR* and Δ*virS* deletion mutants to properly infect Caco2 cells raised the question of whether the expression of listerial internalin A is reduced. This protein has been shown to be critical for cellular adhesion and internalization into Caco2 cells by binding to the glycoprotein E-Cadherin (102). We therefore verified expression of InlA and InlB expression in both the Δ*virR* and Δ*virS* mutant by performing immunoblot analysis. Despite the strong variation of infection into Hela versus Caco2 cells, there were no significant differences detectable in internalin levels using monoclonal antibodies (data not shown). To determine the infection step that is inhibited in the Δ*virR* and Δ*virS* strains, bacteria were stained using an ActA-directed monoclonal antibody during *in vitro* infection of the epithelial cell line PTK2. By means of immunofluorescence microscopy we found fewer bacteria in the host cells but no apparent defect in adhesion or phagosomal escape during infection.

3.2.2.3. Role of the D-alanylation of teichoic acids for immune activation

In *S. aureus* the substitution of D-alanine on teichoic acid has been shown to also be important for cytokine induction (107). In *Lactobacillus plantarum* the deletion of *dlt* significantly reduces cytokine release in a Toll-like receptor 2 (TLR2)-dependent manner. Nevertheless, studies performed in our lab with Δ*dltB* and Δ*virS* mutants of *L. monocytogenes* could not confirm this effect in Hela cells transfected with TLR2 (data not shown). There were no significant differences in TLR2-dependent NF-κB activation induced by *L. monocytogenes* wild type versus the deletion strains.

3.3. Lipoproteins of *Listeria* and Host Response

3.3.1. Background

Recent studies have highlighted the role of lipoproteins as the dominant immune-activation factor of in gram-positive bacteria (17, 62). A deletion mutant of *L. monocytogenes* lacking the prolipoprotein diacylglyceryl transferase gene (*lgt*) was characterized (7) that is unable to transfer the diacylglyceryl moiety from phosphatidyl-glycerol to generate the modified prolipoproteins. Consequently, membrane retention of unmodified lipoprotein precursors is lost. Baumgärtner and colleagues showed that translocation, however, is not

affected since signal peptide removal can still be performed by lipoprotein-specific signal peptidase II (Lsp) (7). Related studies have demonstrated that lipoproteins are essential for the virulence of *Streptococcus* and *Staphylococcus* species (17, 62, 119, 146). For *Streptococcus* it was shown that *lgt* is not essential for cell growth *in vitro* but is essential for viability during infection. Similarly, a transposon-induced *lgt* mutant of *S. aureus* elicits a decreased immune response in host cells following infection due to the absence of mature lipoproteins.

An additional goal of this work involved determining the cell wall components responsible for immune recognition of *L. monocytogenes* by the host. We therefore used the Δ*lgt* deletion strain of *L. monocytogenes* EGDe (7) to study the role of lipoproteins for survival within the host.

3.3.2. Role of lipoproteins in *L. monocytogenes* for immune response and virulence

3.3.2.1. Complementing Δ*lgt* in *L. monocytogenes* EGDe and generating an *L. innocua* Δ*lgt* deletion strain

To examine the ability of the *L. monocytogenes* Δ*lgt* mutant strain to activate host cell responses, a complemented strain using the *L. monocytogenes* site-specific phage integration vector pPL2 harboring *lgt* and its promoter region was produced. Unlike the mutant strain that releases non-lipidated prolipoproteins to the culture supernatant, *lgt* complementation restores the profile of secreted proteins to one resembling that of the wild-type EGDe strain (Fig. 37). This complemented strain was therefore used in the immune response and growth assays described below.

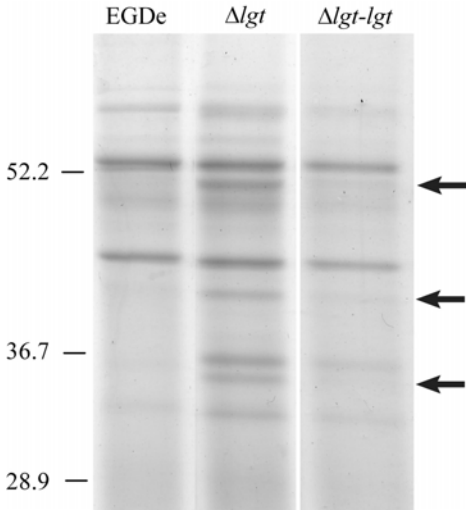

Figure 37: Release of unprocessed proteins into the culture supernatant. Cultures of *L. monocytogenes* EGDe, Δ*lgt* and the complemented strain Δ*lgt*-*lgt* were grown to exponential phase and the secreted proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue. The deletion mutant displays an increased number of bands (arrows) compared to the wild type EGDe and complemented strains that correspond to unprocessed lipoproteins released into the supernatant.

In a variety of bacteria, lipoproteins have been shown to function as pathogen-associated microbial pattern (PAMP) that can activate TLR2 signaling cascades (79, 112). In *L. monocytogenes*, lipoproteins are known to be associated with virulence although a direct link to TLR2-dependent pathways has not been demonstrated (130). To determine whether TLR2 recognition is a primary feature of only the pathogenic *L. monocytogenes* or if it is a general characteristic of the genus *Listeria*, an *L. innocua lgt* deletion strain (LIR Δ*lgt*) was constructed and its ability to induce TLR2 mediated NF-κB activation was examined. The generation of the mutant was performed via standard homologous recombination techniques within the *L. innocua* background. *L. innocua* is non-virulent and incapable of invading epithelial cells, such as the Hela cell line, or to grow in macrophages. Thus the strain is suitable for assessing immune responses independent of its virulence properties. As detailed below, both Δ*lgt* strains, *L. monocytogenes* and *L. innocua*, were examined for their ability to induce an NF-κB based luciferase reporter following *in vitro* infection of human TLR2 (hTLR2)-expressing Hela cells. Additionally, their ability to induce cytokine release in bone marrow-derived mouse macrophages was determined.

3.3.2.2. Lack of diacylglycerol-modified lipoproteins abolishes TLR2-mediated NF-κB activation

TLR2-dependent recognition of *L. monocytogenes*, *L. innocua* and their isogenic *lgt* deletion mutants was investigated by monitoring NF-κB activation in cells co-incubated with these bacteria using the Dual-Luciferase Reporter Assay System. Cell stimulation was performed with either bacterial cells or culture supernatants on transfected Hela cells transiently expressing the firefly luciferase gene under the control of an NF-κB dependent promoter. Co-transfection of a plasmid constitutively expressing *Renilla* luciferase was used for normalization of luciferase activity. To assess TLR2-dependent activity, cells were additionally transfected with either a CMV-promoter driven plasmid expressing hTLR2 or an empty CMV-promoter vector. Relative activity of firefly to *Renilla* luciferase was calculated and the fold change of the normalized signal for stimulated versus non-stimulated cells was determined. Both wild-type strains, *L. monocytogenes* EGDe and *L. innocua* (LIR), showed strong induction of NF-κB-dependent luciferase activity mediated by recognition of TLR2 (Fig. 39). This effect is specific for the TLR2-expressing cells, as no differences between the wild-type and mutant strains are apparent in cells bearing the empty vector. Also, TLR2 recognition is sufficient for NF-κB activation regardless of bacterial virulence or adhesion

properties. The induction could be observed with either wild-type bacterial cells or culture supernatant (Fig. 38A, B). In contrast, incubation with bacterial cells or culture supernatant of the Δ*lgt* deletion strains did not lead to activation of NF-κB. Furthermore, treating culture supernatants derived from *L. monocytogenes* EGDe with lipoprotein lipase decreased the stimulatory signal in a dose-dependent manner (Fig. 38C). By reintroducing the *lgt* gene into the *L. monocytogeneș* Δ*lgt* mutant, the induction levels of NF-κB-dependent luciferase activity were restored to those of the wild-type EGDe strain. Taken together, these data suggest diacylglycerol-modified lipoproteins are responsible for TLR2-mediated NF-κB activation.

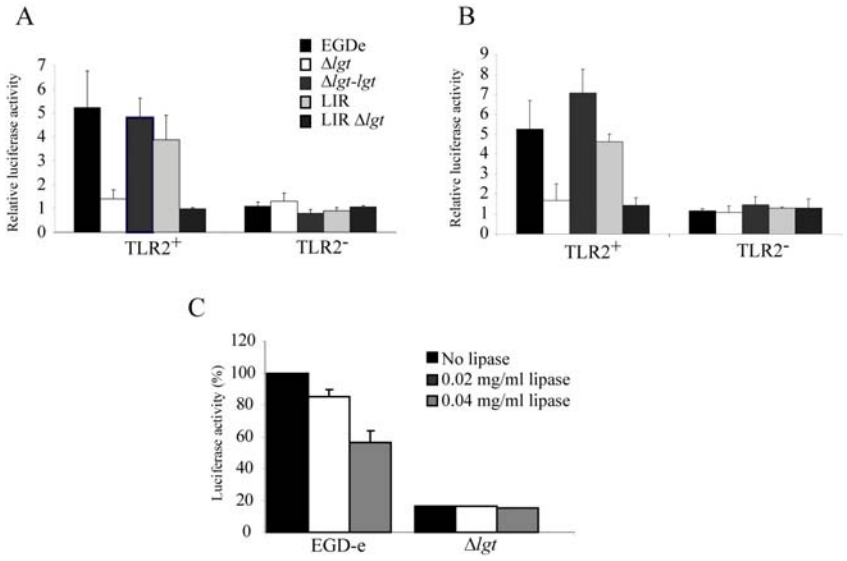

Figure 38**:** NF-κB activation in wild-type *L. monocytogenes* (EGDe) and *L. innocua* (LIR) and their corresponding Δ*lgt* deletion mutants. Fold changes denote stimulated versus non-stimulated luciferase activity in Hela cells transiently expressing human TLR2, an NF-κB firefly luciferase reporter construct (pELAM-luc) and a *Renilla* co-reporter vector (phRL-TK). Stimulation was performed for 5 h with **(A)** bacterial cells or (**B)** culture supernatants of EGDe, Δ*lgt*, Δ*lgt-lgt*, LIR and LIR Δ*lgt*. **(C)** Culture supernatants of *L. monocytogenes* EGDe were treated with streptococcal lipoprotein lipase.

3.3.2.3. TLR2 is required for early recognition of *L. monocytogenes*

Having demonstrated the involvement of lipoproteins in TLR2-mediated NF-κB activation, we next examined the role of lipoproteins in TLR2-dependent inflammatory responses. To this end, cytokine induction in mouse bone-marrow macrophages (BMM) derived from C57Bl/6 wild type and TLR2-deficient mice was examined by co-incubation with the wild type *L. monocytogenes* EGDe and *L. innocua* strains or their respective Δ*lgt* isogenic mutants. As expected, both parental strains activate BMM to induce the pro-inflammtory cytokines TNFα and IL-6 (Fig. 39). In contrast, the levels of cytokine induced

by the Δ*lgt* deletion strains were strongly reduced at early times following infection (4 h and 8 h). However, at 12 hours post infection there was no difference in the levels of cytokines produced seen with the Δ*lgt* deletion strains and their isogenic wild types. Re-introduction of the *lgt* gene by complementation of the *L. monocytogenes* Δ*lgt* mutant restored cytokine levels to that seen with the wild type EGDe strain.

When BMMs derived from TLR2$^{-/-}$ mice were infected with either wild-type strains or their isogenic Δ*lgt* mutants, induction of cytokines was virtually absent at 4 h and 8 h post infection however proinflammatory cytokine responses were observed for all strains at 12 h post infection. Combined, these results indicate that TLR2-mediated recognition is required for the early detection of *L. monocytogenes* during infection. Therefore, either absence of the TLR2-receptor on the host cell or that of the TLR2-ligand (i.e. lipoproteins) on the surface of bacteria has the same result; namely, a delay in the immune responses mounted against thebacteria.

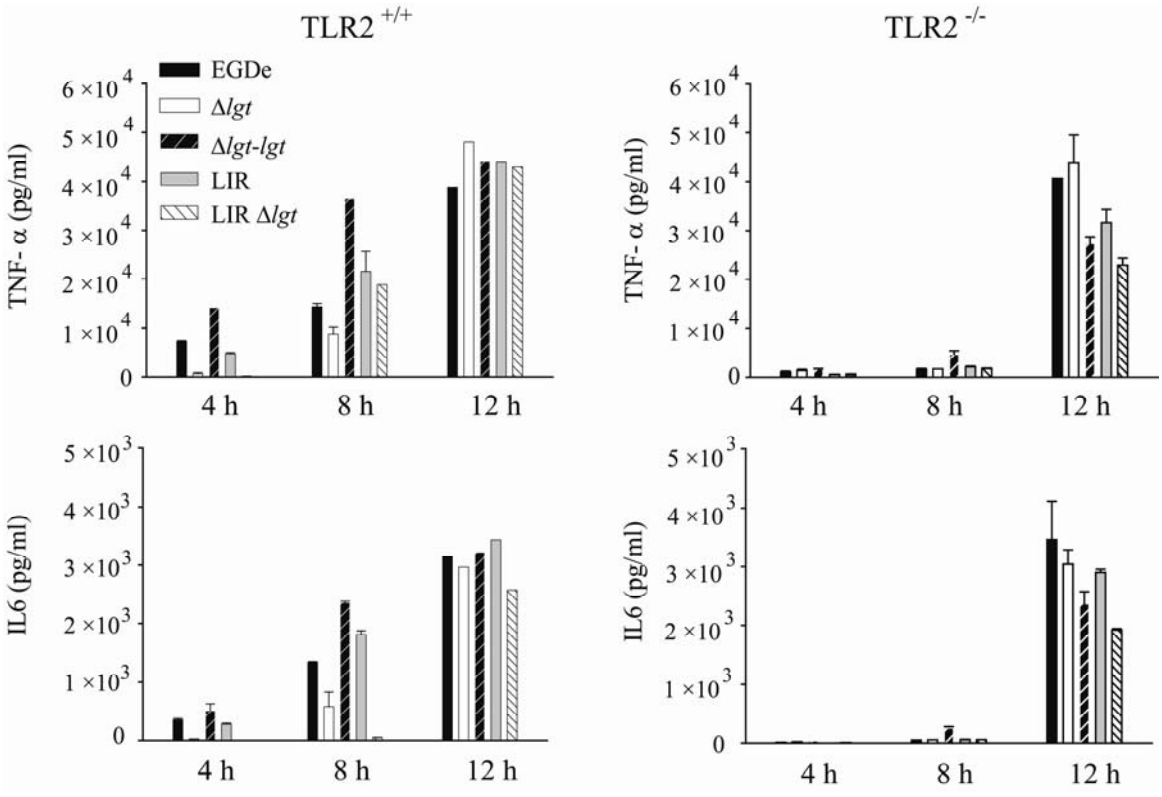

Figure 39: Production of proinflammatory cytokines in bone marrow macrophages derived from TLR2$^{+/+}$ and TLR2$^{-/-}$ mice after stimulation with EGDe, Δ*lgt*, Δ*lgt-lgt* , LIR and LIR Δ*lgt* strains. Cytokine levels of IL6 and TNF-α were measured using the Multiplex cytokine assay in cell culture supernatants harvested at 4, 8 and 12 hours post infection. (Data derived from S. Tchatalbachev, Medical Microbiology, Giessen)

3.3.2.4. The Δ*lgt* mutant is attenuated *in vivo*

The delayed cytokine response found in macrophages infected with the Δ*lgt* mutant strain raised the question of the effects of lipoprotein deficiency on bacterial virulence and survival within the host. We infected C57Bl/6 mice and their TLR2-deficient counterparts intravenously and quantified the bacterial loads in the liver and spleen at day 3 post infection. The wild type EGDe strain showed higher colonization of both the spleens (3-fold) and livers (1.5-fold) of the TLR2-deficient mice as compared to organs of the wild-type mice (Fig. 40).

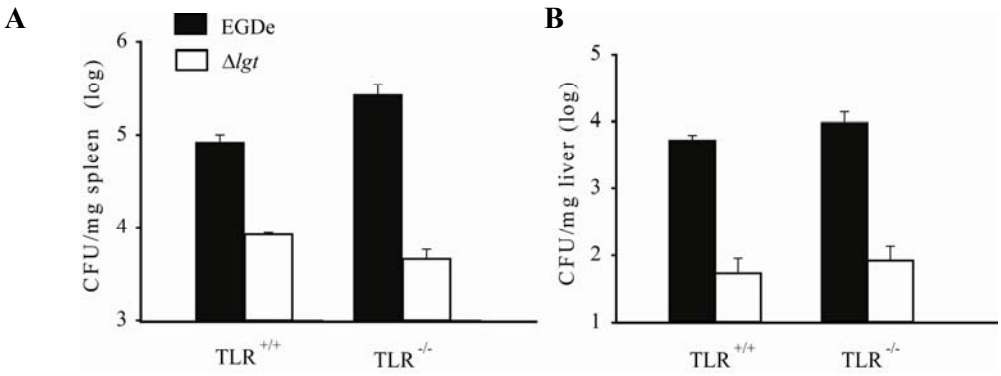

Figure 40: Control of *in vivo* infection by TLR2. C57Bl/6 wild type and TLR2-deficient mice were intravenously infected with EGD-e and Δ*lgt* bacteria (2×10^5 CFUs) and bacterial loads in **(A)** spleen and **(B)** liver were determined on day 3 post infection.

Since the observed attenuation of the Δ*lgt* mutant was independent of the TLR2 phenotype, we quantified bacterial loads of both the Δ*lgt* mutant and the wild-type strain in liver and spleen of BALB/c mice for five consecutive days post infection. The study revealed that although the bacterial numbers of the parental *L. monocytogenes* EGDe strain, at every single time point examined, significantly exceed that of the Δ*lgt* mutant over the five day period examined, the kinetics of organ colonization by bacteria are similar for both strains (Fig. 41A and B). Thus, it is the reduction in the numbers of mutant bacteria during early infection that is largely responsible for attenuation and suggests an intrinsic defect in the ability of these bacteria to either reach and/or to colonize target organs.

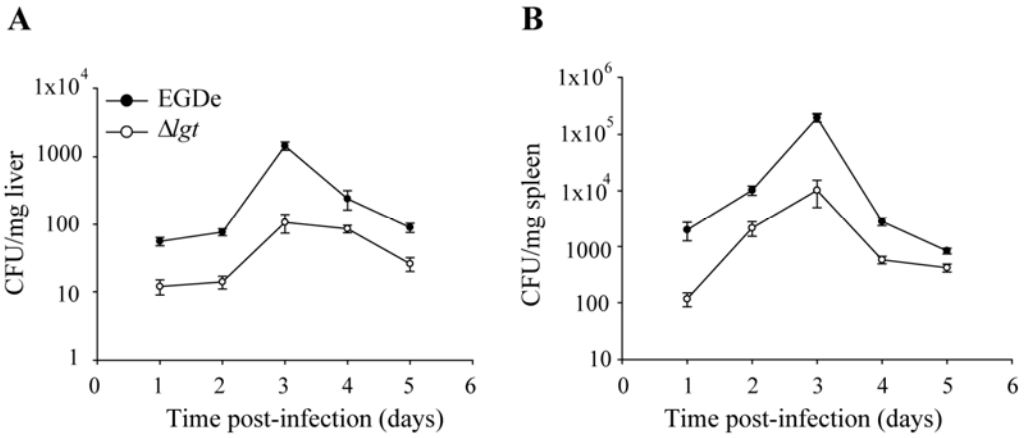

Figure 41: *In vivo* virulence in the mouse model of infection. The kinetics of bacterial growth was followed over 5 days in **(A)** liver and **(B)** spleen of BALB/c mice intravenously infected with EGDe or Δ*lgt* bacteria (2×10^3 CFUs).

The ability of these bacteria to survive in the peripheral blood of infected mice was therefore examined. The levels of wild-type bacteria in blood were over thirty times higher than that of the mutant strain after 6 h of infection with 380 versus 12 bacteria per ml ($p = 0.0648$). At 24 h post infection, there was exuberant growth (> 500 fold) of wild-type bacteria as compared to the Δ*lgt* mutant (10,000 versus 17 bacteria per ml, $p = 0.0540$). The lowered numbers of viable mutant bacteria in peripheral blood after 24 h appear to account for the decreased numbers in the organs of infected mice.

The contribution of lipoproteins to listerial virulence was verified by infecting BALB/c mice with a 100-fold increased bacterial dose (2×10^5 CFU) to test for survival of animals during a 10 day period. As shown in Figure 42, mice infected with the Δ*lgt* mutant survived the 10 days but all animals infected with the EGDe parental strain died within 5 days.

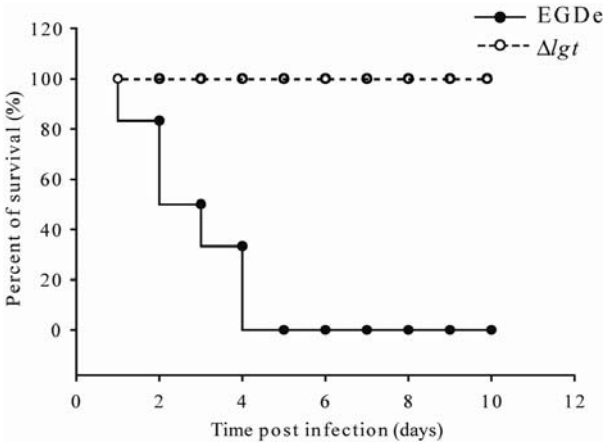

Figure 42: Contribution of lipoproteins to listerial virulence in mice. The survival of BALB/c mice was monitored over a 10 day period after intravenous injection of a lethal dose of bacteria (2×10^5/animal).
(The figure was created by W. Mohamed, Medical Microbiology, Giessen).

3.3.2.5. The Δ*lgt* mutation affects entry and survival in epithelial cells and macrophages

Apart from being poorly bacteremic, deficiencies of the *L. monocytogenes* Δ*lgt* mutant observed in the mouse infection model can also result from additional factors, including problems with adhesion, internalization, escape from the phagolysosome or cell-to-cell spreading. To narrow down these alternatives, we performed *in vitro* assays that allowed us to distinguish between the different stages of infection. We first examined the ability of the deletion mutant to invade non-phagocytotic epithelial cell lines. The invasive capacity of the Δ*lgt* mutant is found to be strongly impaired with infection rates of only 10% or 20% that of the parental EGDe strain in either Caco2 or Hela cell lines, respectively (Fig. 43A).

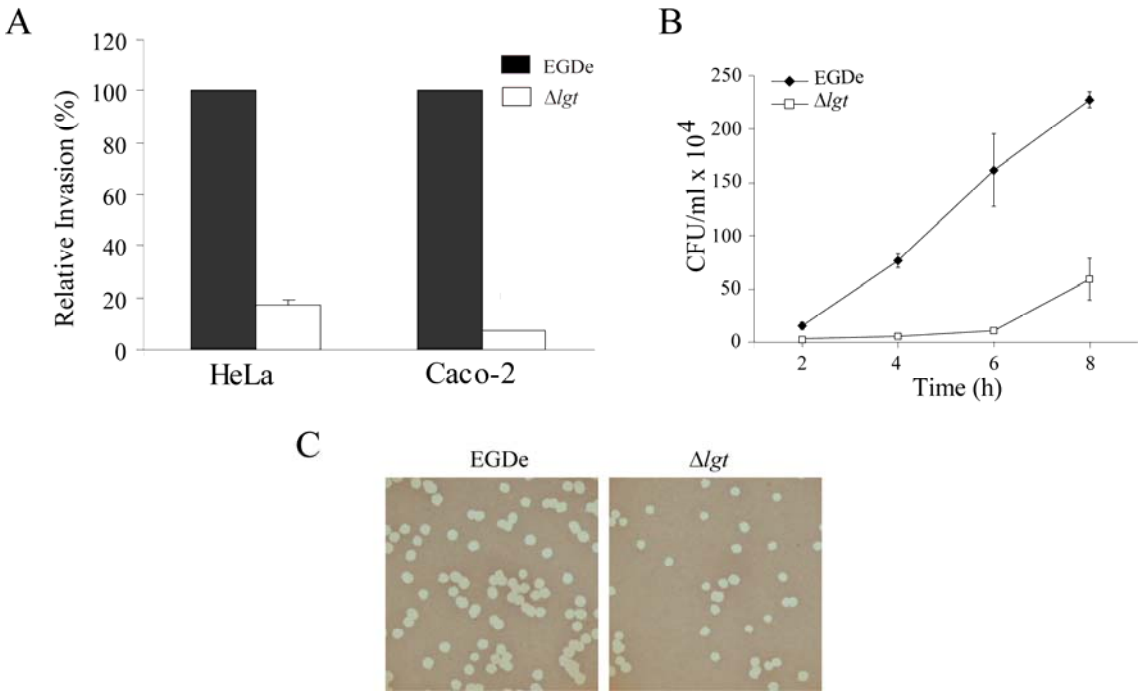

Figure 43: Intracellular survival of *L. monocytogenes* EGDe and Δ*lgt*. Cells were infected with either the wild type EGDe or mutant Δ*lgt*, lysed at indicated times and intracellular bacteria plated in serial dilution on BHI plates. **(A)** Invasive properties of the EGDe or Δ*lgt* mutant strains monitored 2 h after infection. **(B)** Growth of intracellular bacteria in P388D1 murine macrophages. **(C)** Plaque-forming assay. Monolayers of L-929 cells were infected for 4 h, incubated for 4 days at 37°C and plaques visualized with neutral red.

Next, potential intracellular-growth defects were assessed by infecting P388D1 murine macrophages with wild type EGDe or the Δ*lgt* mutant and then measuring intracellular bacterial counts at indicated time-points post infection. Whereas growth of the deletion strain is greatly reduced in early infection, with up to 15-fold less CFUs compared to wild type at 6 h (Fig. 43B), growth of the Δ*lgt* mutant is apparent at 8 h, indicating a general ability of the bacteria to grow intracellularly. Finally, using a plaque-forming assay, which

examines both intracellular growth and cell-to-cell spread, we found that while the plaque size is essentially indistinguishable between the mutant and parental EGDe strain (Fig. 43C), the total number of plaques formed by the Δ*lgt* strain is ~ 40% lower. The plaque size between the mutant and parental EGDe strain differed by about 20%.

3.3.2.6. Lack of lipoproteins renders *L. monocytogenes* sensitive to cationic peptides

The important and varied roles of lipoproteins for the gram-positive envelope have been discussed in detail by Sutcliffe and Russell (150). As these functions can involve antibiotic resistance as well as substrate binding and transport, we wondered if the lack of membrane-bound lipoproteins in the Δ*lgt* deletion strain would also render *Listeria monocytogenes* sensitive to cationic antimicrobial peptides (CAMPs). Therefore, growth in the presence of Gallidermin, Polymyxin B or Colistin was evaluated for the parental EGDe, the Δ*lgt* mutant and the complemented Δ*lgt*-*lgt* strains (Fig. 44). Whereas the deletion mutant exhibits no growth defects when cultured in BHI only, in media supplemented with antimicrobials growth of the Δ*lgt* strain is reduced compared to the wild type EGDe strain. This deficiency can be alleviated by restoring the *lgt* gene, as evidenced by the growth characteristics of the complemented Δ*lgt*-*lgt* strain. Notably, this sensitivity to antimicrobials correlates with the decreased virulence of the bacteria as revealed in the *in vitro* and *in vivo* experiments outlined above.

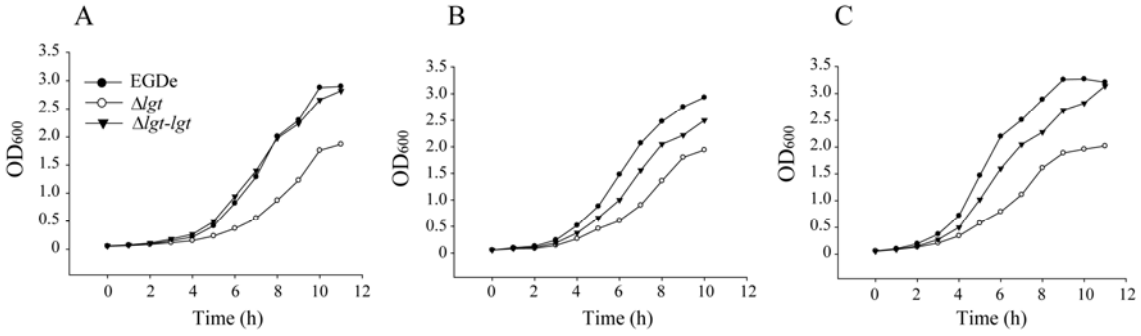

Figure 44: Susceptibility to cationic peptides. The *L. monocytogenes* EGDe, Δ*lgt* and Δ*lgt*-*lgt* strains were treated with **(A)** 32 µg/ml Colistin, **(B)** 0.5 µg/ml Gallidermin or (**C**) 20 µg/ml Polymyxin B. Optical density (OD_{600}) of growing bacteria was recorded as indicated.

Various lipoproteins in *L. monocytogenes* have been identified using the Δ*lgt* mutant. Among those are OppA and PrsA, proteins that are up-regulated during cold-stress (unpublished data) and are required for bacterial growth at low temperature. Therefore the *Listeria* strains lacking *lgt* were tested for sensitivity at cold temperatures. The deletion

mutant was previously shown to have wild type-like growth properties at 37°C in BHI broth (7). In contrast, growth rates of the Δ*lgt* mutant were clearly diminished at 20°C and 8°C as shown in Figure 45.

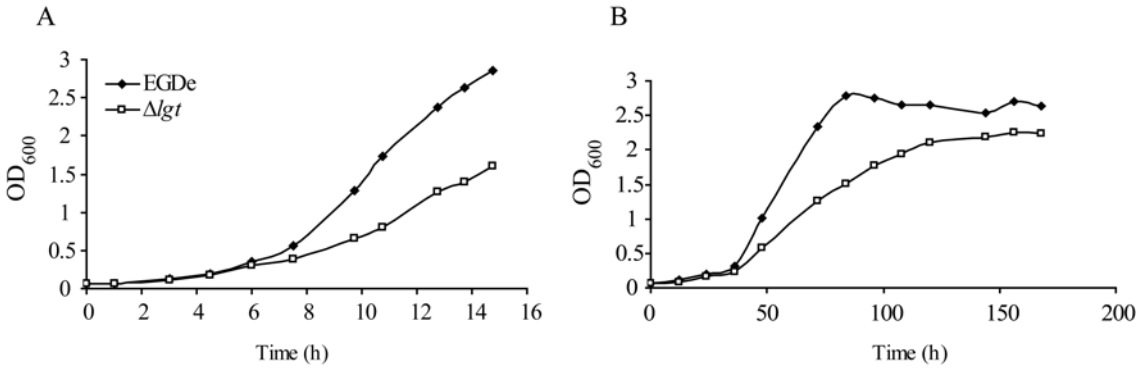

Figure 45: Bacterial growth of *L. monocytogenes* EGDe wild type and the Δ*lgt* mutant at low temperature. Bacteria were cultured in BHI broth at **(A)** 20°C and **(B)** 8°C and growth was monitored via the optical density at 600 nm.

A similar effect was seen when the swarming behavior of the *lgt* deletion strain was tested using a cultivation temperature of 20°C. The flagellar motility of Δ*lgt* was 80 % that of the parental strain, implying a defect in growth at the lowered temperature rather than a defect of flagellar expression. A strong inhibition in growth of the Δ*lgt* mutant strain was also observed under ethanol stress (Figure 46).

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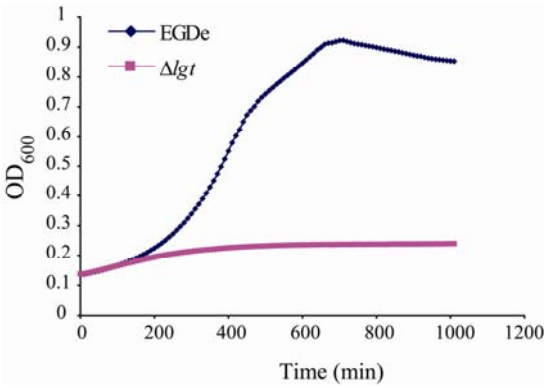

Figure 46: Growth of the *L. monocytogenes* EGDe wild type and the Δ*lgt* mutant in BHI supplemented with 5% ethanol. Bacterial strains were shaken at 37°C and growth was determined by automated measuring of the optical density at 600 nm using the infinite series 200 plate reader (Tecan).

4. Discussion

4.1. Murein hydrolases in *L. monocytogenes*

4.1.1. Transition of a smooth to rough phenotype depends on two autolysins, MurA and p60

The role of murein hydrolases for the survival and pathogenesis of the bacterial cell has been the focus of various studies over the past years. Several autolytic enzymes, among those Ami and p60, were found to be involved in cell adhesion or/and actin-mobility during the infection process (82, 103, 122). Penicillin-binding protein homologues have also been shown to contribute to antibiotic resistance, cell morphology, and virulence of *Listeria monocytogenes* EGDe (56). The phenotypic smooth-rough transition has recently been associated with a SecA2 secretion system that is responsible for translocation of two autolysins, p60 and MurA, and 17 other listerial proteins (89). We reasoned that an examination of existing rough mutants of *L. monocytogenes* would allow us to pinpoint if and which of the autolysins are involved in this transition. The p60 autolysin is one of the most prominent listerial proteins found in the culture supernatant of *L. monocytogenes* EGDe, with smaller amounts of p60 also detected on the bacterial surface. MurA seems to be more strongly attached to the bacterial cell wall because it is not released to the culture supernatant as observed with p60. Both proteins contain LysM motifs responsible for attachment to the bacterial cell wall. The number of LysM domains might influence the strength of cell wall attachment since MurA contains four whereas p60 only contains two of these modules.

This study reports the simultaneous reduction in protein level of p60 and MurA in several rough variants of *L. monocytogenes*. The data indicate that both proteins are in some way either diminished, absent, or impaired in various strains with rough colony morphology. The apparently inactive, truncated form of p60 in the rough strain SLCC7506 illustrates the important role of the NlpC/p60 catalytic domain for proper protein function. Although this truncated p60 was expressed *in vivo* in quantities similar to that of wild type, implying stable protein production, the rough phenotype is indicative of a loss in activity. In the case of the other mutants, a relocation of the p60 protein from the culture supernatant to the membrane was detected.This suggests a connection between the necessity for efficient transport of p60 out of the cell and formation of the smooth phenotype, with the rough strains perhaps having an impaired SecA2 protein. However, sequencing of the *secA2* gene of two of the rough

variants (SLCC7509, SLCC7510) investigated here revealed no evidence of changes either in the promoter region or the SecA2 protein itself. Since alleles of the *secA2* gene cloned from several rough variants successfully complemented the Δ*secA2* mutant strain, it seems unlikely that a truncation or reduced expression of the SecA2 protein would be responsible for the defect. Nevertheless, defects in other gene products responsible for the proper function of the SecA2 secretion system might be involved. Unlike p60, MurA, the second SecA2 dependent hydrolase in *L. monocytogenes,* did not show intracellular accumulation in any of the rough variants or in the Δ*secA2* deletion mutant. As such it may be that an increased level of intracellular MurA is concomitant with increased degradation. The zymographic analysis of various rough isolates shows that three strains (SLCC7504, SLCC7506, SLCC7509) release increased amounts of undefined lytic proteins as shown by additional bands in supernatant fractions. The role of these additional hydrolases in the culture supernatant of these isolate still remains unclear.

The reduced extracellular level of p60 in rough variants of *L. monocytogenes* was previously described (82, 89, 132) and, until the successful generation of a Δ*iap* deletion mutant by Pilgrim *et al.* (122), was thought to be solely responsible for the rough phenotype (168). Data presented in this work agree with the observation of Pilgrim and colleagues (122) that additional factors, in conjunction with the p60 reduction, are needed. The Δ*iap* deletion mutant generates chains but does not display the rough colony morphology. Similarly, disruption of the *murA* gene alone does not convert the phenotype from smooth to rough. During growth the Δ*murA* deletion mutant exhibits long chains in exponential phase that fall apart to individual cells when the cultures reach lag phase (24). The filaments of the Δ*murA* mutant are distinct from those of the rough mutants because the chains show a clear constriction between each cell. In contrast, septum formation in strains lacking p60 is only visible via transmission electron microscopy, which shows that constriction of cell poles is inhibited. MurA thus seems be important for the separation of individual cells at a late stage in cell division whereas p60 is involved in cleavage at an earlier phase. Indeed, studies with *E. coli* have previously shown that *N*-acetyl-muramyl-L-alanine amidases (e.g. MurA) are required at the terminal stages of septation (63, 64). The target of both murein hydrolases on the bacterial cell still remains unclear. Localization of peptidoglycan hydrolysis is essential because uniform bacterial shape has to be maintained during cell division. The presence of repeat domains is considered relevant for localization on the bacterial surface. For instance, the Atl autolysin of *S. aureus* harbors three repeats that have been shown to direct the protein

to the equatorial surface ring (4). Binding studies using recombinant protein tagged with fluorescent markers would be useful to obtain information about localization of p60 and MurA on the bacterial surface.

Lenz and colleagues (89, 90) characterized a rough *L. monocytogenes* strain that resulted from the deletion of a second *Listeria secA* gene associated with protein secretion. They discovered that autolysins p60 and MurA (NamA), as well as various other secreted and surface-associated proteins, are dependent on the SecA2 secretion system. These SecA2-dependent proteins were assumed to be involved in the formation of long cell chains and rough colony morphology in *L. monocytogenes*. By creating the Δ*murA* Δ*iap* double mutant, we were able to identify two specific gene products responsible for the smooth to rough phenotype conversion. Mutation of each single gene, however, only causes the formation of shorter cell chains without rough colony morphology. Only the simultaneous lack of both proteins in Δ*murA* Δ*iap* or the Δ*secA2* deletion strain results in the smooth-rough transition.

Rough *Listeria* strains have so far only been identified in the *L. monocytogenes* species. Since the investigated hydrolases and the SecA2 secretion system are also present in *L. innocua* and *L. welshimeri*, the question arises whether it is possible to alter the phenotype of these species from smooth to rough. The role of an additional SecA secretion system for pathogenic strains has been discussed extensively by Lenz and colleagues (90) and was suggested to play a role in host colonization. The identification of a secondary SecA secretion system in both pathogenic and nonpathogenic *Listeria* strains implies a common role for this system. Genes homologous to p60 and MurA were identified in the genomes of other *Listeria* species also having the SecA2 system. The dependence of both autolysins on the SecA2 system may be linked to the *secA2* gene being conserved in those strains.

4.1.2. Rough strains have a reduction in both biofilm formation and flagellar motility

The role of the smooth-rough colony transition in the lifecycle of *L. monocytogenes* remains unclear. There are numerous different rough phenotypes that vary in chain length, colony morphology, septum formation, pathogenicity and motility. The smooth-rough transition in *L. monocytogenes* not only influences its pathogenicity but also leads to altered colonization features. *L. monocytogenes* does not generate capsules, thus a connection between rough colony morphology and variability in capsular material can be excluded (161). Rough isolates of *L. monocytogenes* were previously shown to give enhanced biofilm formation on stainless-steel coupons within a bioreactor system (105). In contrast, rough

strain variants presented in this study had an overall decreased ability to form biofilms. A similar effect was found with the rough Δ*murA* Δ*iap* double mutant giving rise to decreased colonization of plastic wells. Flagellar motility is reduced with both rough strains Δ*murA* Δ*iap* and ΔsecA2. A more moderate reduction in sessile growth was observed with single deletion of *iap* and *murA*, respectively. Surprisingly, a much higher decrease in flagellar motility could be observed with the single deletion mutants Δ*iap* and Δ*murA* (30% vs > 60% decrease). This was somewhat unexpected as one would predict that strains lacking both p60 and MurA would be even less motile than strains lacking each protein individually. One reason for this discrepancy may lie in the method of how flagellar motility is determined. The extent of swarming is monitored in soft agar plates by measuring the area of growth. Since colonies of the rough phenotype appear bigger than those with the smooth phenotype on normal BHI agar plates, the growth area in the soft agar plates might be larger independent of its swarming properties.

4.1.3. Strains lacking p60 and MurA are attenuated in virulence

Diverse rough *Listeria* strains demonstrate decreased pathogenicity by reduced invasion in mouse fibroblast cells (82) and in mouse models (133). In this context, the decreased virulence of rough bacterial strains that generate long filaments could conceivably be the result of an increased size and/or altered shape that inhibits effective adherence and uptake into the eukaryotic cell. Hence, infection experiments with rough strains are not appropriate in determining the role of putative gene products for virulence. Nevertheless, recent data demonstrate the importance of the *iap* or *murA* gene products for proper invasion by generation of single deletion mutants (93, 122). The Δ*iap* deletion strain was also found to be impaired in cell to cell spreading due to defective actin polymerization (122). This defect arises from inhibited cell division due to the absence of p60 preventing polarization of ActA. Attenuation of the Δ*iap* or Δ*murA* deletion mutants was increased with double mutation of both genes, leading to very low invasion and replication rates within various host cells (93). As stated above, the participation of the altered rough cell morphology in Δ*murA* Δ*iap* that might partly lead to the dramatic virulence defects has to be considered with these infection experiments. Promotion of listerial pathogenesis by p60 and MurA has also been shown using the Δ*secA2* deletion strain that was rapidly cleared after systemic infection of murine hosts and had reduced cell to cell spread (89). The SecA2 system was described as an auxiliary secretion system conserved in several pathogenic gram-positive bacteria including *S. aureus*,

Mycobacterium tuberculosis, *Bacillus anthracis* and virulent strains of *Streptococcus pneumoniae* (89). The authors proposed that SecA2-dependent secretion has evolved in part to promote the secretion of autolysins with important contributions to *L. monocytogenes* virulence. However, we found that SecA2 as well as p60 and MurA are also present in apathogenic *Listeria* strains, contradicting the authors hypothesis of SecA2 being the gram-positive equivalent of a specialized secretion pathway (e.g. Type I-IV) known for gram-negative bacteria.

4.1.4. Combined deletions of MurA or p60 with other murein hydrolases, Ami, Auto and p45 do not change morphology

Cell chains formed by the mutated EGDe strains Δ*iap*, Δ*murA*, Δ*murA* Δ*iap* and Δ*secA2* highlight the importance of both autolysins p60 and MurA for cell division. That additional autolysins other than p60 and MurA might be involved in the transition to a rough phenotype is suggested by the observation that some rough strains do not have a decreased extracellular p60 level (133). In these cases, impaired synthesis or expression of other murein hydrolases was proposed to be responsible for the rough phenotypes, having otherwise normal p60 and MurA expression levels. The necessary participation of more than one cell wall hydrolase likely plays an important role in the generation of a rough phenotype in *L. monocytogenes*. Indeed, a similar situation is seen in *Bacillus* where a combined deficiency of several murein hydrolases increases the length of cell chains (70, 96, 97, 113). By simultaneous deletion of *murA* or *iap* with each of three genes encoding the previously described murein hydrolases Ami (*ami*), Auto (*aut*) and p45 (*spl*), we tried to generate *L. monocytogenes* mutants with rough colony morphology similar to the Δ*murA* Δ*iap* strain. None of the double mutants presented a rough phenotype, however, they resembled the morphology of the Δ*murA* and Δ*iap* single mutants.

A further point of interest was to determine the interplay of the characterized murein hydrolases with each other and its effect on the bacterial cell. Proteins of the NlpC/p60 super-family exhibit functional diversity and are widely represented in various bacterial lineages (2).

4.1.5. Deletion of *spl* leads to enhanced autolysis and to increased sensitivity to ethanol

The protein p45 belongs to NlpC/p60 super-family and has been shown to have peptidoglycan hydrolyzing activity (140). It shares 55% similarity and 38% identity with p60

of *L. monocytogenes* and was proposed to have similar functions (140). The protein contains a 27 amino acid putative signal sequence but none of the common modules for cell attachment such as LysM motifs, GW modules or LPXTG domains. Nevertheless, it has been identified as a member of the cell wall subproteome of *L. monocytogenes* by serial protein extraction followed by 2-D PAGE and mass spectrometry analysis (137). How the protein is tethered to the bacterial surface remains unknown. So far there have been no studies with mutation strains of p45 in *L. monocytogenes*. By generating the p45 deletion mutant Δ*spl* we found that the strain is not defective in cell division similar to the Δ*iap* mutant, despite the similarities between p60 and p45. Unexpectedly, the Δ*spl* deletion mutant displayed a dramatic increase in autolysis compared to the wild type EGDe, implying that p45 does not promote but rather prevents autolysis in the bacterial cell. Even though p45 has been previously shown to have peptidoglycan lytic activity (140), we found no difference in the lytic profile between the deletion strain and the wild type using *Micrococcus*, *Listeria* and *Bacillus* substrates. One reason might be that the protein could not be extracted by the methods used here for fractionation of bacterial proteins. Previous studies with p45 revealed that only small amounts of the protein are secreted and the authors used a highly concentrated and purified fraction for zymogram analysis to demonstrate a weak lytic band with *Listeria* substrate (140).

The Δ*spl* deletion mutant showed no prominent phenotype regarding growth in BHI broth at various temperatures but is more sensitive to vancomycin and ethanol compared to the wild type EGDe. The susceptibility towards ethanol has been observed for all five single deletion mutants lacking murein hydrolases (p60, MurA, Ami, Auto, p45). The strongest impairment was found with strains that are without p45 or p60. Both proteins harbor the NlpC/p60 domain with proposed peptidase activity. Ethanol is thought to influence integrity of biological membranes by reduction of hydrophobic interactions between phospholipid acyl chains in the bilayer (15, 68). The composition of the cell membrane plays a crucial role for resistance against ethanol. Increased content of unsaturated fatty acids has been shown to improve bacterial growth under ethanol stress conditions (68). Altered expression of certain membrane proteins by modifications on the bacterial surface might therefore account for decreased membrane fluidity and decreased tolerance towards ethanol.

The lowered tolerance for vancomycin that inhibits cell wall biosynthesis indicates changes on the bacterial surface. The large hydrophilic molecule is able to form hydrogen bond interactions with the terminal D-alanyl-D-alanine moieties of the NAM/NAG-peptides.

This binding of vancomycin to the D-Ala-D-Ala prevents the incorporation of the NAM/NAG-peptide subunits into the peptidoglycan matrix. The general resistance mechanisms, mainly found in enterococci, appear to be alteration to the terminal amino acid residues of the NAM/NAG-peptide subunits. Variations such as D-alanyl-D-lactate and D-alanyl-D-serine decrease the affinity of vancomycin dramatically. The influence of p45 on vancomycin tolerance is highly speculative here. Since the hydrolase acts as a peptidase cleaving peptide bonds between the amino acids it might promote assembly of alternated terminal residues. However, these variations are only known for *Enterococcus* species as vancomycin therapy is considered the last line of defense against serious infections caused by gram-positive organisms such as enterococci, MRSA and *Clostridium difficile*. Assembly and degradation of murein are closely connected as new cell wall material can only be integrated after hydrolases have cleaved the rigid outer layer. The murein hydrolase p45 might therefore also promote resistance against vancomycin by inducing overproduction of target sites in the outer layers of the peptidoglycan. This could have the effect that the antimicrobial drug does not reach the important target sites where the wall is assembled on the outer surface of the cytoplasmic membrane. Cell wall synthesis and turnover have been shown to be up-regulated in vancomycin-resistant staphylococci strains leading to thicker and more disorganized cell walls (145). The altered bacterial cell walls were proposed to have reduced affinity to vancomycin and can trap the antibiotic at the periphery of the cell (145). Similar effects might also be caused by the absence of p45 leading to changes on the bacterial surface and thereby blocking the action of vancomycin.

Unlike mutation of *iap* or *murA* the deletion of *spl* does not affect the virulence properties of *L. monocytogenes*. In Hela cells only, double mutation of *spl* with *murA* and *iap* resulted in higher invasion rates than obtained with the EGDe wild type. The double mutation of *spl* with either *murA* or *iap* did not, in general, increase deficiencies seen with the single mutants Δ*murA* and Δ*iap* but resembled their phenotype or that of Δ*spl*. One exception could be observed with susceptibility towards cell wall antibiotics. The double deletion of *spl* with *murA* caused increased sensitivity against some cephalosporins that was not seen with the single mutants lacking either *spl* or *murA*. *L. monocytogenes* has been shown to have intrinsic resistance against these ‘modern’ cephalosporins (159). The increased sensitivity by simultaneous lack of both murein hydrolases, MurA and p45, indicates that these enzymes act synergetically to generate the natural resistance. Hydrolysis of the -OC–N- lactam bond of the β-lactam ring is a common resistance strategy carried out by so-called β-lactamases. How

the intrinsic resistance against certain cephalosporins is achieved in *L. monocytogenes* remains unclear. For the gram-negative bacterium *E. coli* a β-lactamase gene *ampC* was shown to be induced by the peptidoglycan degradation product GlcNAc-1,6-anhydro-MurNAc peptide (tripeptide) (148). It is possible that in *L. monocytogenes* certain cephalosporin resistance genes are also induced by cell wall degradation products obtained by the combined activity of MurA and p45. Even though the Δ*iap* Δ*spl* deletion strain lacks two peptidases, the deficiencies seen with the single Δ*spl* or Δ*iap* mutants do not accumulate. The fact that the bacterial morphology of the Δ*spl* mutants and the growth rate under rich media conditions resemble that of the wild type indicate that p45 is redundant for the cell division process under normal growth conditions. Nevertheless, the enhanced susceptibility to ethanol and vancomycin as well as increased autolysis implies that activity of this protein is necessary to promote optimal growth under stress conditions.

4.1.6. Absence of Ami leads to defects in cell-to-cell spread

The Ami protein is found on the bacterial surface but not in culture supernatants of *L. monocytogenes* (103). It contains a 30 amino-acid putative signal sequence, a 179 amino-acid N-terminal alanine amidase domain and a C-terminal domain made up of four repeats of approximately 160 amino acids, each containing two GW modules. Studies with the Ami amidase of different serotypes of *L. monocytogenes* revealed that whereas its N-terminal enzymatic domain is highly conserved, the C-terminal cell wall anchoring (CWA) domain is variable (104) thus enabling attachment to lipoteichoic acids (LTA) on the bacterial cell wall. For example, the Ami protein of the serotype 1/2a (i.e. EGDe) has eight GW modules, whereas that of serovar 4b contains only six (104). Milohanic *et al.* showed that the CWA domain mediates bacterial binding to mammalian cells and that Ami 4b binds HepG2 human hepatocytic cells less efficiently than Ami 1/2a (104). The number of GW modules was therefore considered to influence adhesion to mammalian cells. Surprisingly, overexpression of Ami 1/2a CWA caused decreased entry into HepG2 cells but not to Caco2 cells (103). Although binding to Caco2 human enterocytic cells is similar for Ami 1/2a and Ami 4, in this case the association between bacteria and the cell surface may be too strong and the entry process may be hindered. Previous studies with mutant strains of *L. monocytogenes* disabled in Ami production demonstrated deficient invasion in the human liver cell line HepG2 but not in other cells tested such as Caco2 and SK-Mel 28 (103). InlB, the essential protein for invasion of epithelial cell lines (i.e. Hela), fibroblasts and hepatocytes also carries GW

modules. These motifs link the protein to the bacterial surface and in addition mediate binding to host ligands. The significance of autolysins being adhesins is also revealed by the presence of two staphylococcal autolysins, AtlE and Aas, with adhesive properties (103).

In this work, we found reduced entry rates by the *ami* deletion mutant in both epithelial cell lines Caco2 and Hela yet normal intracellular growth rates in macrophages. Interestingly, deletion of *ami* also caused impaired cell-to-cell spreading as indicated by reduced plaque sizes in L-929 fibroblasts and by decreased actin-based motility in PTK2 fibroblasts. The Δ*ami* mutant bacteria exhibit less and shorter actin tails although their cell morphology does not differ from the wild type EGDe. This effect is rather interesting because retardation in actin polymerization is often connected to dysfunctional cell division (as in the case of *iap* and *murA*). As explained earlier, polarization of ActA has been proposed to be a direct consequence of the differential cell wall growth rates along the bacterium and is considered to be dependent on the relative rates of protein secretion, protein degradation and bacterial growth (128). The impairment of actin polymerization in the Δ*ami* mutant could therefore result from an imbalance in one of these processes due to the deficiency of Ami. Another explanation for the impaired ability to form actin tails would be the direct participation of Ami in the process of actin polymerization. Recruitment of actin requires the involvement of several eukaryotic factors initiated by ActA binding to VASP. It is possible that Ami is involved in actin binding by interaction with host molecules through its GW domains. The questions of if and how Ami facilitates the polymerization of cellular actin could be addressed in future studies. For instance, testing the interaction of wild type Ami protein and of GW mutants with host molecules required for actin polymerization could yield insight into the nature of the deficiencies.

Simultaneous mutation of *ami* with *iap* showed dramatic impairment in pathogenicity with respect to cell-to-cell spread, intracellular replication in macrophages and invasion into epithelial and fibroblast cell lines, similar to the Δ*murA* Δ*iap* double mutant. Double mutation of *ami* with *murA* results in decreased infection of fibroblasts and macrophages, but does not affect invasion to the higher extent as previously seen with Δ*murA*. The capability of Δ*murA* Δ*ami* to spread from cell to cell is completely lost, as shown by immunofluorescence microscopy. The dramatic difference between individual single mutants and strains lacking both proteins, Ami and MurA, in their ability to form actin tails and to replicate in macrophages displays how efficiently these enzymes can compensate for each other in these

two processes. Altogether these data suggest that simultaneous deficiency of Ami with either p60 or MurA leads to accumulation of defects observed for single deletion mutants.

4.1.7. Deficiency of Auto leads to defects in cellular entry

Another autolysin identified in *L. monocytogenes* is Auto, a putative N-acetyl-glucosaminidase that has been detected in cell-wall associated protein fractions but not in culture supernatants (20). The 62 kDa protein contains a 26-amino-acid signal sequence, a 169 amino-acid N-terminal autolysin domain and a C-terminal domain with four GW modules of approximately 81-83 amino acids (20). Interestingly, the *aut* gene is in a locus comprising several genes presumably involved in TA synthesis, some of which are absent in *L. innocua*. For instance, the adjacent gene *lmo1077* encodes a protein involved in biosynthesis of teichoic acids. Whether these genes products are required for association of Auto with the cell wall is at present unknown. Previous studies demonstrated that Auto is involved in virulence of *L. monocytogenes* similar to other autolysins. Mutation of *aut* was reported to cause decreased entry in various eukaryotic cell lines by Cabanes *et al.* (20). The authors showed that the *aut* mutant strain of *L. monocytogenes* exhibits wild type-like cell-to-cell spreading and intracellular replication but is attenuated in a mouse infection model. These data were verified in this study, wherein a Δ*aut* mutant had strong invasion defects in epithelial cells and normal infection properties in macrophages and fibroblasts. Along with Ami, this autolysin of *L. monocytogenes* harbors GW modules implicated with pathogenicity.

Simultaneous lack of *aut* and *murA* leads to strong defects in bacterial fitness and virulence of *L. monocytogenes*. The double mutant Δ*murA* Δ*aut* was shown to be highly attenuated, with poor infection of epithelial cells and fibroblasts and no intracellular growth in macrophages. The double-deletion strain displays much larger deficiencies in virulence than seen with the isogenic single mutants Δ*murA* or Δ*aut*. For instance, invasion rates into Hela cells go down from ~ 50% to 6% (Table 2). Despite its smooth colony morphology, the double mutant shows attenuation in virulence comparable to the rough Δ*iap* Δ*murA* deletion mutant. The Δ*murA* Δ*aut* strain is also strongly impaired in its ability to form biofilms to a similar extent to that observed with the rough strain Δ*iap* Δ*murA*. This implies that the filamentous cell morphology is not the crucial factor for altered surface attachment but rather the lack of murein hydrolases leading to changes in the bacterial cell wall. MurA and Auto both have the proposed function as N-acetyl-glucosaminidases. The prominent changes in the phenotype found in *L. monocytogenes* lacking two glucosaminidases reveals how important

the interaction of these enzymes is for proper cell function. These data imply that MurA and Auto can compensate for each other in certain functions. The results indicate that the severe attenuation of *L. monocytogenes* is caused by concomitant lack of several murein hydrolases independent of a rough phenotype. In contrast, no significant changes were observed between Δ*iap* and the Δ*iap* Δ*aut* double mutant. This observation implies that p60 and Auto do not share functions but work independently of each other.

4.2. Newly identified putative murein hydrolases

The important roles of previously studied autolysins for biological function and virulence raised our interest to identify more proteins with similar functions in *L. monocytogenes*. Using a bioinformatic approach, proteins of *L. monocytogenes* were selected that contain domains found in cell wall hydrolases or that contain similar cell wall adhesion motifs. Deletion mutants were generated to establish the role of these proteins for *Listeria*.

4.2.1. Deletion mutant Δ*0129* shows decreased intracellular growth

The gene *lmo0129* encodes a putative Ami-like amidase and is localized on a genetic region encoding bacteriophage proteins. The putative murein hydrolase is devoid of any obvious signal peptide but has been detected in *Listeria* supernatant fractions (158). The fact that *lmo0129* is adjacent to a gene encoding a putative holin of the TcdE family, Lmo0128, could be an indication for protein export by the holin-like pathway, which is common for bacteriophage lysins. These endolysins are cell wall hydrolases that are synthesized late during virus multiplication and gain access to their peptidoglycan substrate with the aid of small membrane-disrupting holin proteins mediating the release of progeny virions. Microarray studies of Chatterjee (2006) demonstrated that the expression of bacteriophage genes and of *lmo0129* is up-regulated in the Caco2 epithelial cell line (28). Deletion of *lmo0129* caused a delay in multiplication in macrophages at 6 and 8 hours post infection (Fig. 30). However, immunofluorescence analysis and invasion of epithelial cells showed no obvious changes in comparison to the wild type. A possible role of *lmo0129* in virulence of *L. monocytogenes* remains disputable as the protein contains no common protein domains involved in host interaction.

4.2.2. Lmo1216 is involved in bacterial invasion of Hela cells

Among the group of putative N-acetyl glucosaminidases, such as MurA and Auto, are two proteins encoded by *lmo1215* and *lmo1216*. Both genes are presumably transcribed individually as each gene has its own putative promoter. Downstream of *lmo1217* is a gene encoding a protein similar to endo-1,4-beta-glucanase and to aminopeptidase. The genes upstream of *lmo1215* have unknown function. Phenotypic changes of the Δ*1215* and Δ*1216* deletion strains were not detected with regard to cell morphology, indicating that these putative hydrolases are not involved in cell division. The protein profile of cell wall hydrolysis showed no alternations between the wild type and deletion mutants. However, both genes were suggested to be involved in virulence of *L. monocytogenes* based on microarray studies with Caco2 infected cells (28). It was noted that *lmo1216* gene expression is up-regulated in bacteria inside the vacuole and *lmo1215* gene expression is up-regulated in cytosolic bacteria (28). In infection experiments presented here, the Δ*1216* mutant exhibited a decreased ability to invade Hela cells but not Caco2 cells (Table 4). Intracellular growth in P388D1 macrophages and actin polymerization within PTK fibroblasts was not found to differ between Δ*1216* and the wild type. Deletion of the *lmo1215* gene did not show any effects on virulence of *L. monocytogenes*. The decreased invasion of Δ*1216* could be reasoned by the presence of a GW module, which has previously been linked to host entry of *L. monocytogenes* (see Ami or Auto). However, this study showed that absence of other listerial proteins harboring GW modules (e.g. Lmo1215, Lmo1521 and Lmo2591) has no effect on the virulence of the pathogen. Mouse infection studies would be necessary to confirm a role of *lmo1216* for pathogenesis. The creation of a double mutant Δ*1215* Δ*1216* could also be useful to determine if the proteins compensate each others function. As seen with previously studied double mutants lacking *iap* and *murA*, simultaneous deficiency of several genes can accumulate defects seen with single mutants and even lead to new phenotypes.

4.2.3. The strains Δ*0717* and Δ*1521* have impaired flagellar motility

The *lmo0717* gene encodes a protein containing an *N*-acetyl-muramidase domain and is located in a genetic region encoding proteins involved in flagellar biosynthesis. Interestingly, this study showed a 30% decrease in flagellar motility of the *L. monocytogenes* strain lacking the Lmo0717 protein. This observation suggests that the putative murein hydrolase assists the murein turnover to enable assembly of bacterial flagella. The assertion is

supported by the finding that a recently described transcriptional repressor, MogR, regulates *lmo0717* together with other genes located upstream that are associated with flagellar assembly (143). However, our data suggest that activity of Lmo0717 might be assisted or adopted by other murein hydrolases because the Δ*0717* deletion strain did not lose swarming ability entirely; namely, the negative control, a Δ*flaA* deletion mutant, displayed a more pronounced defect in motility (over 70%) compared to the EGDe wild type. Besides the impaired motility, there were no phenotypic changes in morphology or virulence of the Δ*0717* strain compared to its parental strain. The protein encoded by the *lmo0717* gene therefore seems to have no role in cell division or host invasion of *L. monocytogenes*.

Lmo1521 is another putative hydrolase that could be involved in the assembly of bacterial flagella. The deletion of *lmo1521* results in lowered swarming in soft agar plates to levels similar as seen with Δ*0717*. Lmo1521 belongs to the group of amidases like Ami and it contains two N-terminal GW modules that may be required for surface attachment. Besides the defects in flagellar motility, the Δ*1521* deletion mutant presents no other phenotypic anomalies compared to the EGDe wild type.

4.2.4. Lmo1104 exhibits increased sensitivity to cell wall antibiotics and has reduced host invasion

The only deletion mutant of putative cell wall hydrolases that demonstrated variations in antibiotic resistance was Δ*1104*. The deletion strain was shown to be more susceptible to two types of cephalosporins, antibiotics that act like other β-lactams as inhibitors for cell wall biosynthesis. The lack of Lmo1104 appears to modify the bacterial cell envelope in ways that make it less tolerant towards these bactericidal antibiotics. As proposed above, the Lmo1104 protein might have a dual function in *L. monocytogenes* since it carries two hydrolytic domains, a C-terminal NlpC/p60 domain and an N-terminal lytic *N*-acetyl-muramidase domain. Murein hydrolases with dual function have also been found in other bacteria. Recently, a two-domain autolysin, termed CwlT, has been described in *B. subtilis* with both *N*-acetylmuramidase and endopeptidase activity (45). The role of this murein hydrolase in the bacterial remains unknown. Interestingly, Lmo1104 is the only examined protein in this study that is found exclusively in *L. monocytogenes*, a fact that piqued our interest in the pathogenicity of the deletion strain. Cell to cell spreading and intracellular replication are not affected by deletion of the *lmo1104* gene. Infection studies of epithelial cells with the Δ*1104* deletion mutant, however, showed significant changes in invasion of Hela cells compared to

the EGDe wild type. The impaired ability of the mutant to efficiently invade the epithelial cell line implies that Lmo1104 is somehow involved in invasion by *L. monocytogenes*.

4.2.5. Lmo1941 and Lmo1303, LysM proteins of unknown function

Although the putative Lmo1941 protein containing a LysM motif has no unknown function there are homologies to a peptidoglycan-binding protein, YpbE, of *Bacillus licheniformis*,. The role of Lmo1941 in the cellular function of *L. monocytogenes* remains unclear. The Δ*1941* deletion strain showed a significant decrease in flagellar motility and a mild susceptibility to ethanol but had no other phenotypic variation compared to the wild type. Similarly, the deletion strain for *lmo1303* did not present phenotypic changes compared to the wild type EGDe besides a lightly decreased tolerance towards ethanol. The listerial gene *lmo1303* is homologous to a gene of *B. subtilis* encoding YneA, which is involved in the SOS stress response by suppressing cell division (76). The authors found that *yneA* is repressed in the wild type cell under normal growth conditions and is induced during SOS response. Artificial expression of the YneA protein has been shown to cause cell elongation. Simultaneous deletion of the *yneA* gene and of *dinR*, which encodes the SOS regulon repressor in *B. subtilis*, compensates the filamentous phenotype of the Δ*dinR* mutant. The listerial *lmo1303* gene is located downstream of *lexA*, a homologue to *dinR* in *L. monocytogenes*. Our attempts to construct a chromosomal knockout mutant of EGDe for *lexA* by using a standard allelic replacement procedure were not successful. However, the genetic location and its homologies to YneA imply that the protein has a similar function for inhibition of cell division during SOS stress response. Studies of Chatterjee *et al.* (2006) demonstrated that gene expression of *lmo1303* is highly up-regulated during infection (29). Activation of Lmo1303 would imply that cell division is halted during host infection. The authors propose that cell division is reduced inside the host cells as they observed down regulation of FtsZ and FtsA, the major bacterial cell division determinants, and down regulation of murein hydrolases such as p60, MurA and p45.

The downregulation of peptidoglycan hydrolase activity might be a strategy by *Listeria* to reduce the amount of bioactive muropeptides that are recognized by the host leading to stimulation of immune responses. The observation by Humann *et al.* (2007) that an *iap* deletion in *L. monocytogenes* leads to decreased activation of pro-inflammatory cytokines supports this hypothesis (66).

4.2.6. Deletion strains are less tolerant to ethanol stress

In contrast to the strong effect that the absence of the previously described autolysins (e.g. MurA, p60, Ami and Auto) has on the bacterial cell, there were only few phenotypic changes seen with the deletion strains lacking other proteins with proposed cell wall hydrolysis function in *L. monocytogenes*. Almost all newly generated mutant strains were more susceptible to ethanol than the EGDe wild type. The inhibitory effect of ethanol varied greatly between the different deletion mutants. The strongest difference was observed with Δ*2522*, deficient in a protein containing two LysM domains. The decreased ethanol tolerance might be due to changes in the cell wall by the absence of hydrolyzing enzymes as described earlier. Only the strain Δ*1521*, lacking a putative amidases, showed wild-type like growth under the influence of ethanol.

4.2.7. Cell- wall hydrolase activity not confirmed

None of the deletion strains lacking putative murein hydrolases displayed altered cell or colony morphology as seen with Δ*iap* or Δ*murA*. Accordingly, the newly described putative cell wall hydrolases probably have no significant role during cell division. Zymogram analysis showed no altered cell-wall hydrolytic activity in any of the deletion mutants compared to the wild type EGDe. The MurA hydrolase gives the most prominent band in lytic gels containing cell wall substrate. None of the lytic bands seen within the gels were absent in any of the deletion mutants for hypothesized cell wall hydrolases. Using Triton-induced autolysis, no significant changes were found between wild type and deletion strains. The expression of the main autolysins of *Listeria*, MurA and p60, was determined by western blot analysis and showed no differences between the mutants and their parental strain. Therefore, we can assume that neither MurA nor p60 compensate for any of the proteins absent in the deletion strains. The data do not address the hydrolase activity of any of the tested proteins with the methods used in this work. To successfully substantiate their function would require the generation of recombinant proteins or gene over-expression. The assignment of a distinct function of peptidoglycan hydrolases is often difficult for many bacteria possess a large number of hydrolases and they seem to have redundant roles (162). In addition, various cell wall hydrolases of *E. coli* are thought to have more than one function, such as the cleavage of the septum during cell division, release of turnover products from peptidoglycan during cell growth and induction of autolysis (63, 64). Like p45, a few of the investigated putative hydrolases lack common surface association motifs such as LPXTG,

GW or LysM. How these proteins, encoded by *lmo0717*, *lmo1104* and *lmo0394*, are linked to the bacterial envelope remains unclear.

4.3. Strategies of resistance to cationic antimicrobial peptides (CAMPs) in *L. monocytogenes*

4.3.1. Immune activation studies with strains deficient in D-alanylation of teichoic acids

Teichoic acids (TAs) and lipoteichoic acids (LTAs) are immunostimulatory components of the gram-positive cell wall. The negatively charged polymers are thought to provide structural support for the cell wall and a biophysical barrier to prevent the diffusion of substances (108). It is also conceivable that they assist in regulation of cell growth by providing binding sites for autolysins and thus limiting their activity. The composition of lipoteichoic acid, in particular the D-alanine content can modulate the immune response, as evidenced by a Δ*dlt* deletion mutant of *Lactobacillus plantarum* that resulted in decreased stimulation of proinflammatory cytokines in peripheral blood mononuclear cells and monocytes (55). A similar effect has been described with synthetic LTAs having L-alanine instead of D-alanine in a *dlt* mutant of *S. aureus*. Additionally, the *dltB* mutant of *L. monocytogenes* was tested for its ability to induce cytokine response in host cells. For this purpose, TLR2-transfected Hela cells were stimulated with EGDe and Δ*dltB* to compare NF-κB activation. Since no significant changes could be found between wild type and the deletion mutant, other factors apart from the TLR2 receptor may be involved in LTA recognition that are absent on Hela cells. Macrophages or human whole blood were used in the previous studies with *Lactobacillus* or *Staphylococcus*, expressing a large repertoire of different classes of pattern recognition receptors and other surface antigens that stimulate cytokine response. The use of macrophages to look at the immunological effect of D-alanylation in *L. monocytogenes* would be expedient for future studies.

4.3.2. Listerial strains Δ*virR* and Δ*virS* are highly susceptible to CAMPs

The VirR/S regulon was examined for its function to control resistance for cationic antimicrobial peptides (CAMPs) in *L. monocytogenes*. For *Salmonella typhimurium*, a two component system, PhoQ and PhoP, was shown to control be a control the induction of resistance mechanisms to host antimicrobial peptides, for example modifications of lipid A of

the lipopolysaccharide (LPS) (57). Upon induction of the PhoQ/PhoP system, the transcription of a large number of genes is initiated, including those that promote antimicrobial peptide resistance. The data presented in this study provide conclusive evidence that VirR/VirS is a two component system with a similar function as PhoQ/PhoP during infection with *L. monocytogenes*. Several genes have been discovered to be controlled by VirR, among those are genes of the *dlt* operon and *mprF* (95). The unmodified teichoic acids of the *dlt* mutants lack D-alanine, resulting in cells that carry an increased negative surface charge. A Δ*dltA* deletion mutant of *L. monocytogenes* has been shown to be more vulnerable to cationic peptides (1). The study showed that deletion of *virR* or *virS* results in strong susceptibility to cationic peptides as demonstrated with the bacteria-derived peptides Polymyxin B, Colistin and Gallidermin and with the human cathelicidin LL37. Growth of the Δ*virR* and ΔvirS strains is completely inhibited under the influence of cationic peptides, whereas growth of the EGDe wild type only shows a mild delay compared to media without antibiotics. As expected, both the Δ*dltB* and Δ*dltC* deletion strains of *L. monocytogenes* EGDe were not able to grow in the presence of cationic peptides. Phenotypes of several bacterial strains with altered cell wall D-alanine content have been reported (1, 100, 114, 153, 165). For example, *S. aureus* exhibits aberrant cell morphology, increased susceptibility to vancomycin and impaired virulence in addition to sensitivity against CAMPs. Deprivation of D-alanine in teichoic acids of *B. subtilis* leads to enhanced autolysis and renders bacteria sensitive to β-lactam antibiotics.

Quantitative real-time PCR was carried out to determine the effect of CAMPs on the expression of "resistance genes" *virR*, *virS*, *dltA* and *mprF*. While the presence of Colistin or Polymyxin B did not change expression of the VirR/S regulon, there was significant up-regulation of *dltA* and *mprF*. As these genes are being controlled by the VirR/S system (95), the regulon presumably functions as a sensoring system for cationic peptides resulting in up regulation of resistance genes.

4.3.3. Decreased virulence of CAMP-sensitive deletion mutants of *L. monocytogenes*

Previous studies in mice showed that the regulatory protein VirR is essential for virulence of *L. monocytogenes* but not the histidine kinase VirS (95). Data presented in this work show that invasion of epithelial cell lines with CAMP-sensitive deletion strains is very much dependson the cell type. While the Δ*virR* and Δ*virS* strains were almost completely impaired in Caco2 cell entry, these mutants showed wild-type like invasion in Hela cells.

Decreased adherence of the Δ*dltA* deletion mutant to Caco2 cells has been previously connected to the increased electronegativity of its surface charge (1). However, this does not address the large discrepancy between the epithelial cell lines. We wondered if the large difference between the two cell types could be explained with altered expression of InlA, a listerial protein required for invasion by binding the E-cadherin of Caco2 cells. However, both internalins, InlA and InlB, were strongly expressed in the CAMP-deficient strains as determined by immunoblot analysis. Altered binding activities of listerial adhesins have also been suggested to be a possible cause for the impaired invasion properties (1). The authors made this proposal based on the observation that alanylation of LTAs can modulate the rate of post-translocational folding of exported proteins in *B. subtilis* by maintaining high concentrations of cations at the membrane-wall interface (67).

The dramatic variation of cell entry between Caco2 and Hela cells could also be explained by diverse expression of CAMPs in different cell lines. While the production of CAMPs has been described for Caco2 cells, to our knowledge there have been no reports regarding the Hela cell-line expression of cationic peptides. The cathelicidin LL-37/human cationic antimicrobial protein 18 (LL37), for instance, is not expressed in epithelial cells of the small intestine but shows strong expression in colon epithelium (60). The human defensins hBD-1 and hBD-2 have also been shown to be expressed in Caco2 cells (111). Previous studies that presented decreased invasion of the Δ*virR/S* strains and Δ*dltA* of *L. monocytogenes* have been carried out with Caco2 cells but not with other epithelial cell lines (1, 95). The high susceptibility to cationic peptides could also explain the deficiency of the CAMP-sensitive strains to propagate in macrophages. However, decreased motility as seen by diminished plaque sizes of Δ*dltB/C* and Δ*virR/S* deletion strains in fibroblasts could also account for reduced intracellular growth.

4.3.4. Deletion of *dal* leads to increased susceptibility to cephalosporins and to ethanol

In this work the role of the enzyme D-alanine racemase, involved in D-alanylation of LTAs, has been studied by generating a Δ*dal* deletion mutant in *L. monocytogenes*. Previous studies with a Δ*dal* Δ*dat* double deletion mutant in *L. monocytogenes* demonstrated that absence of D-alanine leads to attenuation of virulence (153). By generating a double mutant, the authors tried to develop a suitably attenuated form of *L. monocytogenes* that could be used as a safe vaccine and adjuvant (153). Simultaneous deficiency of both genes results in a dependence on exogenous D-alanine in the culture media to enable replication (153). In

contrast, as shown in this study deletion of *dal* alone does not generate this dependency. The Δ*dal* mutant shows no growth defects with CAMPs but is less resistant against cephalosporins and exhibits a strong susceptibility to ethanol. As observed with listerial deletion mutants lacking murein hydrolases, the absence of the D-alanine racemase causes dramatic changes on the bacterial surface leading to decreased tolerance towards these stress factors. Knockout of the alanine racemase gene has also been shown to have dramatic effects in other bacteria, for instance septation defects and cell wall perforation in *L. plantarum* and enhanced cell lysis in *B. subtilis* (114). Deletion of the alanine racemase affects gram-negative bacteria that lack teichoic acids. The increased cell lysis and altered morphology in *E. coli* are mainly attributed to problem in peptidoglycan (PG) biosynthesis (166). D-alanine is incorporated in precursors of PG (UDP-NAM-L-Ala-D-Glu-*m*DAP-D-Ala-D-Ala) as a D-Ala-D-Ala dipeptide, resulting in the formation of tripeptide precursors upon D-alanine starvation. Incorporation of these truncated precursors in PG is thought to reduce cross-linking of glycan strands leading to a weakening of the cell wall (166). Similar effects might lead to the high vulnerability of the *L. monocytogenes* Δ*dal* deletion mutant to ethanol and β–lactam antibiotics. Lack of D-alanylation of teichoic acids probably plays a smaller role in this susceptibility as the Δ*dltB* and Δ*dltC* deletion mutants do not exhibit the phenotype seen with Δ*dal*. These observations illustrate that although an alternative pathway to obtain D-alanine exists in *Listeria* by the activity of D-amino acid aminotransferase, there are considerable changes on the cell surface upon deletion of *dal* alone.

4.4. Lipoproteins are critical for TLR2- dependent microbial recognition and pathogenesis of *L. monocytogenes*

4.4.1. TLR2 detects *Listeria* during early infection by binding released lipoproteins

The data presented in this study unequivocally demonstrate that lipoproteins of *L. monocytogenes* and *L. innocua* are targets for recognition by TLR2 and are independent of listerial virulence properties. Cell-activation experiments with culture supernatant provide conclusive evidence that soluble components released into the environment are sufficient for microbial recognition by host cells expressing TLR2 and for induction of defense mechanisms by activation of NF-κB. Markedly, treatment of wild-type *L. monocytogenes* culture supernatant with lipoprotein lipase caused a decrease of the stimulatory activity in a dose-dependent manner. It is likely that soluble lipopeptides are recognized before the host

cell comes in direct contact with the bacteria. Similar results have recently been reported for *S. aureus* (61, 146) and provide additional support for the significance of TLR2 in recognition of bacterial lipoproteins.

Results presented here show early induction (4 h, 8 h) of the cytokines TNF-α and IL6 in isolated BMM of C57Bl/6 mice with the *Listeria* parental strains but not with their isogenic Δ*lgt* mutants. The deletion strains were nevertheless able to induce wild type-like cytokine levels later during infection (12 h). In contrast, TLR2 deficient macrophages showed only weak induction at early time points (4 h, 8 h) with all strains tested. Despite the absence of TLR2 signaling, high cytokine levels were produced upon longer incubation time (12 h). This implies that TLR-dependent cytokine induction is mediated via binding bacterial lipoproteins and is an early event during infection. The cytokine induction at later time-points also suggests that recognition of PAMPs relies on redundant features and that signal-triggering may necessitate other extracellular receptors of the TLR family. Intracellular sensors of the TLR and NLR family might also be involved in cytokine induction promoted by the Δ*lgt* mutants, given that both wild-type and mutant bacteria are phagocytosed by macrophages. Possible additional candidates for signal-triggering include TLR5 and TLR9, responsible for recognition of flagellin and of non-methylated bacterial DNA, respectively, (79), or NOD1 and NOD2, receptors for catabolic products of peptidoglycan (12, 147). The idea of redundant triggering is supported by studies with knockout mice that lack the common protein adaptor of TLR signaling, myeloid differentiation factor 88 (MyD88) (157). As these mice are more susceptible to infection with *L. monocytogenes* than TLR2-deficient mice, it suggests TLR2 is important for controlling infection but also that other MyD88-dependent signals are required for host resistance.

4.4.2. The importance of lipoproteins for listerial virulence and fitness

A study by Petit *et al*. (119) with a Δ*lgt* deletion mutant of *S. pneumoniae* provided the first insight to the significance of prelipoprotein lipidation for virulence. It was subsequently shown that impaired lipoprotein-processing caused by deleting the lipoprotein signal peptidase gene (*lsp*) causes attenuation in *L. monocytogenes* (130) and *M. tuberculosis* (134). In virulence studies with the mouse model as presented here, similar effects using the *lgt* deficient *L. monocytogenes* strain were found. Transient bacteremia, which is a characteristic of infection with *L. monocytogenes* wild-type bacteria, was almost abrogated in the case of the Δ*lgt* mutant. A reduction in bacterial loads in spleen and liver was evident for

the mutant strain throughout the infection, yet its growth kinetics in both organs were similar to those of wild type (Fig. 39A and B). After the peak of infection and organ colonization on the third day, the wild-type strain was cleared more rapidly than the deletion mutant and the bacterial loads became more alike by day five (Fig. 39A and B). These observations suggest that whereas the Δ*lgt* mutant is defective in adhesion to the host cells and invasion, once inside the host cell, this strain is capable of intracellular replication and cell-to-cell spreading. The reduced virulence properties of the Δ*lgt* strain were verified by performing mouse survival assays with lethal doses of bacteria. The mice infected with the deletion strain survived over a 10 day period post infection but all *L. monocytogenes* EGDe infected mice died within 5 days (Fig. 40). Infection of TLR2 deficient mice results in increased organs loads of EGDe wild-type bacteria, supporting the observation made by Torres *et al*. (157) that optimal control of listerial infection requires TLR2. Bacteria lacking lipoproteins are not only attenuated in wild type mice but also in mice deficient in TLR2 signaling. These data clearly indicate that the immunostimulatory properties of lipoproteins are independent of their contribution to virulence.

The *in vitro* experiments performed in this study provide additional support for the role lipoproteins play in the virulence of *L. monocytogenes*. Invasion and uptake of the Δ*lgt* strain in epithelial and macrophage cell lines, respectively, were noticeably affected. As intracellular growth appears to be delayed rather than completely abolished (see Fig. 41B, growth at 8 h), this suggests that either lipoproteins are not directly involved in this process or that the non-lipidated forms remain active. Nevertheless, several lipoproteins have been shown to take part during the early infection processes of cell entry and phagosomal escape. Among the Lgt-dependent lipoproteins characterized by Baumgärtner *et al.* (7), OppA and LpeA are associated with intracellular survival and bacterial entry (51) whereas the expression of three other lipoproteins is controlled by the virulence regulator PrfA (7).

To further understand the diminished virulence of the Δ*lgt* strain, bacterial resistance against cationic antimicrobial peptides was examined and growth of the deletion mutant was found to be moderately inhibited (Fig. 42). This effect can be rationalized by either the access of these cationic peptides to the cell membrane being facilitated in the absence of lipoproteins or the anchored lipoproteins contributing to the overall charge of the bacterial surface. These data imply that lipoproteins are important for virulence by affording increased resistance against these microbial defense factors. While the exact mechanism is unknown, it is very

likely that antimicrobial peptides produced by host cells during infection are factors in the reduced survival of the Δ*lgt* strain *in vivo*.

Interestingly, Bubeck-Wardenburg *et al*. (17) presented data on an Δ*lgt* deletion strain of *S. aureus* that proliferated to a higher extent compared to wild type during mouse infection. They demonstrated that *S. aureus* variants lacking lipoproteins are able to escape activation of an innate immune response and therefore survive better within the host. In contrast, the *in vivo* studies presented here reveal that the Δ*lgt* deletion strain of *L. monocytogenes* is attenuated in virulence. Unlike Staphylococci*, Listeria* rely on an intracellular replication cycle to disseminate and propagate during infection, given that extracellular *Listeria* are cleared relatively quickly by resident macrophages and circulating leucocytes. Several factors, among them lipoproteins such as PlcB, LpeA, and PrsA are important for the invasion of non-phagocytic cells and persistence in the host (29, 58, 130). It would thus appear that the *Listeria* Δ*lgt* deletion strain, which lacks lipoproteins and has a decreased ability for cell invasion, is prevented from entering the relatively secure intracytosolic environment and therefore shows reduced survival. Clearly, depending on the type of bacterium studied, the absence of TLR2-dependent recognition can have drastically different consequences for virulence.

Besides the impaired virulence the Δ*lgt* deletion mutant also exhibited deficiencies in biological fitness. Due to the absence of lipoproteins such as OppA, an oligopeptide-binding protein required for bacterial growth at low temperature (13), the Δ*lgt* deletion strain shows reduced growth rates at cold temperatures and reduced flagellar motility. In addition, lack of lipoproteins on the surface of *L. monocytogenes* leads to increased susceptibility to ethanol. This is not surprising given that the composition of the cell membrane is crucial for resistance against ethanol as discussed earlier. For example a decrease in unsaturated fatty acids due to missing lipid anchors in the membrane naturally leads to reduced bacterial growth under ethanol stress conditions (68). Taken together, these data convey the significance of lipoproteins in *Listeria* for proper cell function.

4.5. Peptidoglycan binding proteins in *L. monocytogenes*

The functions of various cell wall components in *L. monocytogenes* have been addressed in this study and are summarized in Figure 47.

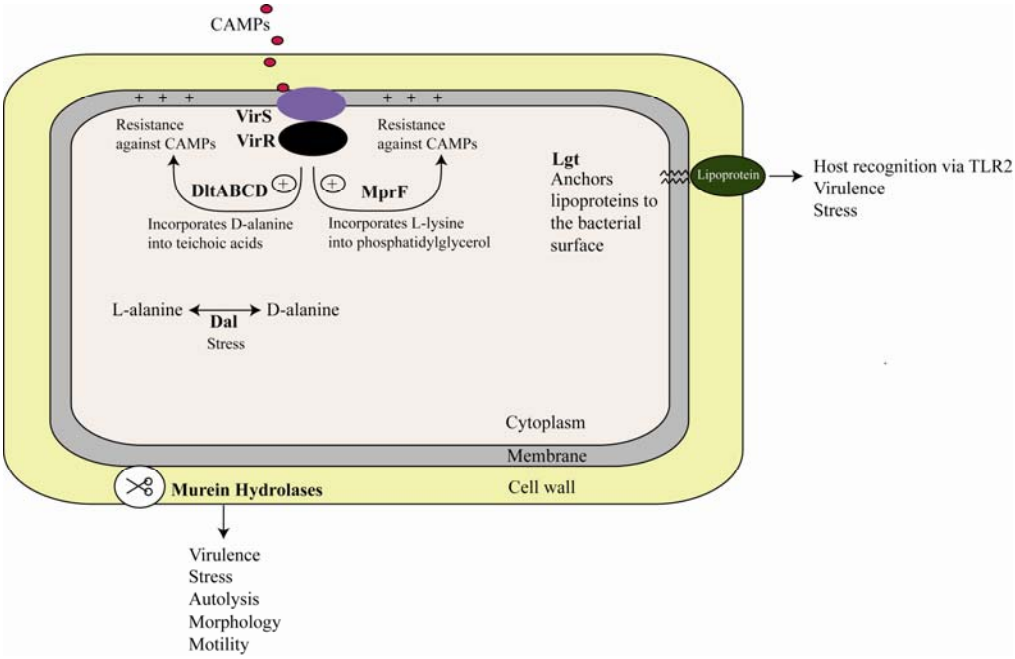

Figure 47: Overview of the proteins studied in this work. The schematic of an *L. monocytogenes* cell illustrates the cellular localization and environmental triggers of key proteins involved in virulence.

Bacterial surface proteins are crucial for interactions with the environment and provide targets for host immune response in pathogenic bacteria. Murein hydrolases cleave the bacterial cell wall and are important for diverse biological processes. The lack of partcicular murein hydrolases has been shown to affect virulence, stress, autolysis, morphology and motility in *L. monocytogenes*. The VirR/S two-component system is suggested to be a regulatory element responding to the presence of CAMPs. Genes of the *dltABCD* operon and *mprF* are activated by VirR, leading to incorporation of D-alanine into teichoic acids and of L-lysine into phosphatidylglycerol in the bacterial membrane. These modifications add positive charges to the bacterial surface and thus provide resistance against CAMPs. The alanine racemase Dal generates D-alanine required for the modification of teichoic acids and for peptidoglycan biosynthesis. Deletion of the *dal* gene has been shown to reduce stress tolerance of *L. monocytogenes*; probably due to impaired cell wall synthesis. The presence of lipoproteins on the bacterial surface is dependent on Lgt, the enzyme required for prelipoprotein lipidation. Data presented in this work reveal the significance of lipoproteins for virulence and stress tolerance as well as host recognition mediated by TLR2.

4.6. Outlook

The roles of the newly identified putative murein hydrolases studied in this work remain unclear and therefore warrant further investigation. The lytic action of these proteins could be examined by generating recombinant vaiants and using them for zymogram analysis with various cell wall substrates or performing cleavage assays with murein sacculi and subsequent analysis of reaction products by HPLC. Analytical methods, such as proton nuclear magnetic resonance, could be used to determine components of purified cell wall derived from cell wall mutants of *L. monocytogenes*. Additionally, it would be of interest to study the regulation systems responsible for the control of murein hydrolases. As these enzymes degrade the cell wall, they must be tightly controlled to prevent autolysis. Identifying the controlling system responsible for the down regulation of murein hydrolases and of other proteins required for cell division during infection would be invaluable. Candidates for such studies include two-component systems or other proteins of proposed regulatory function that are highly expressed during infection.

Cell-wall synthesis of rod-shaped bacteria occurs in two steps, septation and elongation, that are thought to be catalyzed by different protein complexes. The roles of required cell-wall hydrolases and other cell division proteins of *L. monocytogenes* for the two modes of synthesis are still undefined. Localization studies of desired surface proteins in *L. monocytogenes* could be undertaken using fluorescence microscopy with recombinant protein tagged to fluorescent markers (e.g. GFP). Antibiotic drugs such as the fluorescent vancomycin or ramoplanin could also be used for metabolic labeling experiments to illuminate the sites of PG synthesis in living cells. Vancomycin inhibits PG biosynthesis by binding to the terminal D-Ala-D-Ala of cell wall precursors. As most bacteria can rapidly protect themselves from binding of vancomycin by either forming crosslinks within the PG or through hydrolysis of D-Ala by carboxypeptidases, the drug specifically binds to nascent PG (32). In contrast, ramoplanin binds only to the reducing end of the nascent glycan chain found at the initiation sites of PG synthesis and lipid II (155). Both fluorescent antibiotics can be used to probe the pattern of insertion of new cell wall material. Thus, the influence of various proteins associated with peptidoglycan turnover could be examined by using deletion mutants in conjunction with such fluorescence studies. As cytoskeletal proteins have been shown to play a role in directing proteins to the division machinery, the interaction studies could also be performed with murein hydrolases to gain more knowledge about localization and function of individual autolysins.

The decreased virulence properties of the *virR/S* deficient *Listeria* strains also deserve further investigation. Studies with animal models, for instance knockout mice lacking genes encoding cationic antimicrobial peptides, could be performed to narrow down the factors leading to high susceptibility of the Δ*virR/S* deletion mutants. A mouse knockout mutant lacking a murine homologue of the human cathelicidin LL37 has been generated previously and could be used for *in vivo* experiments. Alternatively, bone marrow macrophages could be isolated from these mice to be used for infection assays since there are a variety of other cationic peptides that are still produced by the animals. The role of the VirR/S regulon as a sensoring system for CAMPs should be verified. Complementations of both deletion strains Δ*virR* and Δ*virS* are obvious starting points for additional studies. If successful, these findings could be further extended by investigating the role of individual protein domains, such as the histidine kinase domain or other conserved regions.

The use of the Δ*lgt* deletion strain in *L. monocytogenes* revealed the importance of lipoprotein processing for virulence and biological fitness. There are 68 proteins that are predicted to be lipoproteins in *L. monocytogenes*. The next logical step would be to determine which of these proteins are responsible for the prominent aberrations of Δ*lgt*. Since many of these lipoproteins have unknown functions, the generation of defined deletion mutants and their characterization would be necessary. Comparative genome analysis could help in narrowing down the number of possible candidates.

Additional studies are needed to examine differences in the trajectories the immune response can take during infection in the absence of known PAMPs. The use of defined bacterial mutants that either bypass or enhance innate immune recognition by members of the TLR- and NLR- family provides us with new tools to understand the contributions of the various PAMP molecules during the course of disease. *L. monocytogenes* has been considered a potential live vaccine vector for the induction of cell-mediated immunity for foreign antigens. Such attempts would benefit from a better understanding of the specific bacterial components that elicit unwanted host immune response. The prominent role murein hydrolases obviously play in maintaining cell integrity and virulence for *L. monocytogenes* makes them interesting targets for further studies. The immunostimulatory properties of autolysins via generation of bioactive peptidoglycan subunits have only recently been investigated. Isolation of murein sacculi from deletion mutants that are unable to produce certain peptidoglycan degradation products could be performed to examine host immune response. Similar to methods used for the Δ*lgt* mutant, the defined expression of pattern

recognition receptors in cells devoid of members of the TLR- and NLR- family could be used to determine immunostimulatory components of mutants lacking autolysins. Immune induction by NOD1 and NOD2 would be worthy of further studies with autolysin deletion mutants since these receptors are known to be involved in recognition of peptidoglycan degradation products. Given that *L. monocytogenes* is an intracellular pathogen, the participation of intracellular pattern recognition receptors is relevant for host immune response.

5. Summary

The peptidoglycan layer of gram-positive bacteria contains various components that are crucial for interactions with the environment and for host invasion. In this study, the gram-positive pathogen *Listeria monocytogenes* was used to study the importance of three cell wall constituents for viability and virulence: the cell-wall degrading murein hydrolases (autolysins), teichoic acids and lipoproteins.

Numerous reports have previously documented the spontaneous generation of rough strains from otherwise smooth colonies isolated from clinical samples or the environment. Here I identify the molecular basis of this transition by examining two murein hydrolases, MurA (*murA*) and p60 (*iap*), whose activity are critical for this transition. Whereas single mutation of the respective genes results in a smooth phenotype with chains of bacterial cells, double mutation generates rough colony morphology. Both deletion strains, Δ*iap* and Δ*murA*, are highly attenuated in virulence, exhibiting decreased invasion rates and in the case of Δ*iap*, inhibition of intracellular actin tail formation. Combinatory deletions of each autolysin with other known hydrolases in *L. monocytogenes* (Ami, Auto and p45) were assessed for the smooth-rough transition. Deletion strains were generated deficient in *ami*, *aut* and *spl* (encoding p45) both individually as single mutants and in combination with *murA* or *iap* as double mutants. None of these mutants exhibit a rough phenotype or altered cell morphology.

It was observed that strains lacking *murA*, *iap* or *ami* display altered abilities in autolysis, ethanol tolerance, flagellar motility and biofilm formation. As shown in previous studies, deletions of *ami* and *aut* lead to decreased invasion rates in epithelial cells. I determined that the Δ*ami* strain is additionally impaired in actin tail formation in fibroblasts. Furthermore, a novel Δ*spl* deletion strain was described that while not attenuated in virulence, displays high vulnerability toward ethanol stress and exhibits increased autolysis. Bioinformatic analysis of the listerial genome was used to identify proteins with putative murein hydrolase activity. Characterization of respective deletion mutants revealed that none exhibit changes in morphology or in lytic activity. However, all newly generated bacterial strains are more vulnerable to ethanol stress when compared to their parental strain. For some mutants, impaired flagellar motility and decreased host invasion could also be observed.

The second part of my thesis deals with the modulation of teichoic acids via incorporation of D-alanine to resist attack by cationic antimicrobial peptides (CAMPs). The VirR/S regulon of *L. monocytogenes* has been shown to control various genes, among those resistance genes for CAMPs, *dltA-D* and *mprF*. Deletion mutants lacking *dltB*, *dltC*, *virR*,

virS or *dal*, encoding a D-alanine racemase, were generated to determine their sensitivity towards CAMPs and their function in host invasion. Using quantitative RT-PCR, the *dltB* and *mprF* genes were found to be up-regulated in *L. monocytogenes* EGDe in the presence of the cationic peptides Colistin and Polymyxin B. The Δ*virR* and Δ*virS* deletion strains are unable to grow in otherwise sublethal concentrations of various cationic peptides. They display very low invasion rates in Caco2 cells but not in Hela cells, wherein they exhibit wild type-like entry rates. This discrepancy may be due to the different abilities of Caco2 and Hela cells to produce cationic peptides. Similar effects are seen with the Δ*dltB/C* mutants. Deletion of *dal* does not show the phenotype seen with the Δ*virR/S* and Δ*dltB/C* mutants, indicating that the activity of D-alanine racemase is not required for resistance against CAMPs. These data suggest that the VirR/S two-component system is involved in the control of the *dlt* and *mprF* resistance genes when *L. monocytogenes* is in the presence of cationic peptides.

The final section of my thesis examines the role of lipoproteins for listerial virulence and host immune activation. Recently, a deletion strain of *L. monocytogenes* lacking the prolipoprotein diacylglyceryl transferase (*lgt*) gene was generated that is unable to produce lipoproteins due to the loss of diacylglycerol modification activity. The absence of lipoproteins on the bacterial surface renders the strain insensitive to TLR2 recognition and host cells exposed to mutant *lgt* bacteria show highly delayed proinflammatory cytokine production. In light of these results, we speculate that recognition of *L. monocytogenes* by TLR2 is an early event.

Infection studies of wild-type and TLR2-deficient mice demonstrate attenuation of the *lgt* deletion mutant, implying multiple roles of lipoproteins during infection. Further characterization of the Δ*lgt* mutant revealed that it is impaired in both invasion and intracellular survival, and that it exhibits increased susceptibility to cationic peptides and ethanol. Lipoproteins are identified as the immunologically active ligands of TLR2 and as relevant contributors to the pathogenicity at various stages of infection. Targeted deletions of all 68 lipoproteins in *L. monocytogenes* would allow us to examine how individual mutants are involved in infection and would enable us to assign functions to these lipoproteins.

6. Zusammenfassung

Die Peptidoglykanschicht gram-positiver Bakterien besitzt verschiedene Zellwand-komponenten, die für die Interaktion mit der Umwelt und für die Wirtsinvasion benötigt werden. Wir verwendeten das gram-positive Bakterium *Listeria monocytogenes*, um die Bedeutung dreier Oberflächenkomponenten für Überlebensfähigkeit und Virulenz zu untersuchen: Zellwand-abbauende Mureinhydrolasen (Autolysine), Teichonsäuren und Lipoproteine.

In zahlreiche Veröffentlichungen wurde über die spontane Entstehung von rauen Stämmen aus glatten Kolonien berichtet, die aus klinischen Isolaten oder aus der Umwelt isoliert wurden. Durch die Identifizierung von zwei Mureinhydrolasen, MurA (*murA*) und p60 (*iap*), deren Aktivität entscheidend für die Entstehung des rauen Phänotypen ist, wird hiermit die molekulare Grundlage über diesen Wechsel festgelegt. Beide Einzelmutanten, Δ*iap* und Δ*murA*, sind stark in ihrer Virulenz herabgesetzt, was zu verringerten Invasions-raten und, im Fall von Δ*iap,* zur Hemmung der intrazellulären Aktinschweifbildung führt. Hingegen weisen die Einzelmutanten einen glatten Phänotyp auf mit Kettenbildung der einzelnen Bakterienzellen. Aus diesen Daten könnte mal schließen, dass auch die gleich-zeitige Deletion der beiden Autolysine mit anderen bereits bekannten Hydrolasen in *L. monocytogenes* (Ami, Auto and p45) zu einem Wechsel von glatt zu rau führt. Daher wurden Deletionsmutanten hergestellt, denen die Gene *ami*, *aut* oder *spl* (encoding p45) entweder einzeln fehlen oder in Kombination mit *murA* or *iap* als Doppelmutanten. Es stellte sich heraus, dass keine dieser Mutanten einen rauen Phänotypen oder eine veränderte Zellmorphologie aufweist. Jedoch zeigen die Stämme ohne die Gene *murA*, *iap* oder *ami* veränderte Eigenschaften bei der Autolyse, bei Toleranz gegenüber Ethanol, bei Motililtät mittel Flagellen und bei der Bildung von Biofilmen. Wie schon durch frühere Studien gezeigt wurde, führt die Deletion von *ami* oder *aut* zu verminderten Invasionsraten in Epithelzellen, und zudem wurde hier festgestellt, dass Δ*ami* verminderte Aktinschweifbildung in Fibroblasten aufweist. Im Gegensatz dazu ist die hier neu beschriebene Δ*spl* Mutante nicht attenuiert, aber stark anfällig für Ethanolstress und verstärkt autolytisch. In dieser Studie wurde außerdem versucht, durch Suche nach homologen Regionen andere Proteine von *L. monocytogenes* mit Murein Hydrolase Aktivität zu bestimmen und zwölf Kandidaten wurden ausgewählt. Eine Charakterisierung entsprechender Deletionsmutanten zeigte jedoch, dass keine der neu hergestellten Mutanten Abweichungen in der Morphologie, dem lytischen Proteinprofil oder in ihrer Autolyseaktivität aufweist. Hingegen, erwiesen sich fast alle dieser

Bakterienstämme als sensibler gegen Ethanol im Vergleich zu ihrem Wildtypen. Eine Verminderung der Flagellinmotilität und eine Attenuierung der Wirtsinvasion konnte zudem bei einigen Mutanten beobachtet werden.

Der zweite Abschnitt der Doktorarbeit befasst sich mit der Modulation von Teichonsäuren durch den Einbau von D-Alanin, wodurch die Zelle resistenter gegen Angriffe von kationischen antimikrobiellen Peptiden (CAMPs) wird. Es wurde gezeigt, dass das VirR/S Regulon von *L. monocytogenes* verschiedene Gene kontrolliert, unter anderem auch die Resistenzgene gegen CAMPs, *dltA-D* and *mprF*. Deletionsmutanten der Gene *dltB*, *dltC*, *virR*, *virS* und *dal*, welches für eine D-Alanin Racemase kodiert, wurden hergestellt, um ihre Empfindsamkeit gegen CAMPs zu bestimmen und ihre Bedeutung für die Wirtsinvasion zu untersuchen. Durch quantitative RT-PCR wurde festgestellt, dass die Gene *dltB* and *mprF* unter Einfluß der kationischen Peptide Colistin und Polymyxin B hochreguliert werden. Die Δ*virR* und Δ*virS* Deletionstämme sind nicht in der Lage in subletalen Konzentrationen verschiedener CAMPs zu wachsen. Sie weisen zudem sehr geringe Invasionsraten in Caco2 Zellen auf, haben aber ähnliche Invasionsraten in Helazellen wie der Wildtyp. Ähnliche Effekte wurden auch mit den Δ*dltB/C* Mutanten festgestellt. Die Deletion von *dal* führt nicht zu einem Phänotypen wie bei den Δ*virR/S* und Δ*dltB/C* Mutanten, woraus zu schließen ist, dass die Aktivität der D-Alanin Racemase nicht für die Resistenz gegen CAMPs benötigt wird. Diese Daten unterstützen die Annahme, dass das VirR/S Zwei-Komponenten System unter Einfluß von kationischen Peptiden an der Kontrolle der Resistenzgene *dlt* und *mprF* beteiligt ist.

Der letzte Teil meiner Doktorarbeit untersucht die Bedeutung von Lipoproteinen für die Virulenz von Listerien und der Immunaktivierung des Wirtesimmunsystems. Vor kurzem wurde eine Deletionsmutante von *L. monocytogenes* hergestellt, der das Gen für die Prolipoprotein Diacylglyceryl Transferase (*lgt*) fehlt. Dieser Stamm besitzt nicht die Fähigkeit, Lipoproteine herzustellen, da die Diacylglycerol-Modifizierung nicht mehr stattfindet. Das Fehlen der Lipoproteine auf der Bakterienoberfläche macht den Stamm unempfänglich für eine TLR2-Erkennng. Gleichzeitig zeigten Wirtszellen, die der Δ*lgt*-Mutante ausgesetzt waren, eine stark verzögerte proinflammatorische Cytokinausschüttung. Daher vermuten wir, dass die Erkennung von *L. monocytogenes* durch TLR2 ein frühes Ereignis ist.

Infektionsversuche mit Wildtyp und TLR2-defizienten Mäusen zeigen außerdem eine Attenuierung der *lgt* Deletionsmutante und demonstrieren damit, dass Lipoproteine während

der Infektion viele unterschiedliche Aufgaben haben. Eine nähere Charakterisierung der Δ*lgt* Mutante ergab, dass der Stamm ein verringertes Invasions- und Replikationsvermögen besitzt und eine erhöhte Empfindlichkeit für kationische Peptide und Ethanol aufweist. Hiermit wird gezeigt, dass Lipoproteine immunologisch aktive Liganden für TLR2 sind und einen Beitrag zur Pathogenität in verschiedenen Infektionsstadien leisten. Durch Herstellung von gezielten Deletionsmutanten mit den 68 Lipoproteine von *L. monocytogenes* könnte man untersuchen, welche Lipoproteine bei der Infektion beteiligt sind und welche Funktion sie tragen.

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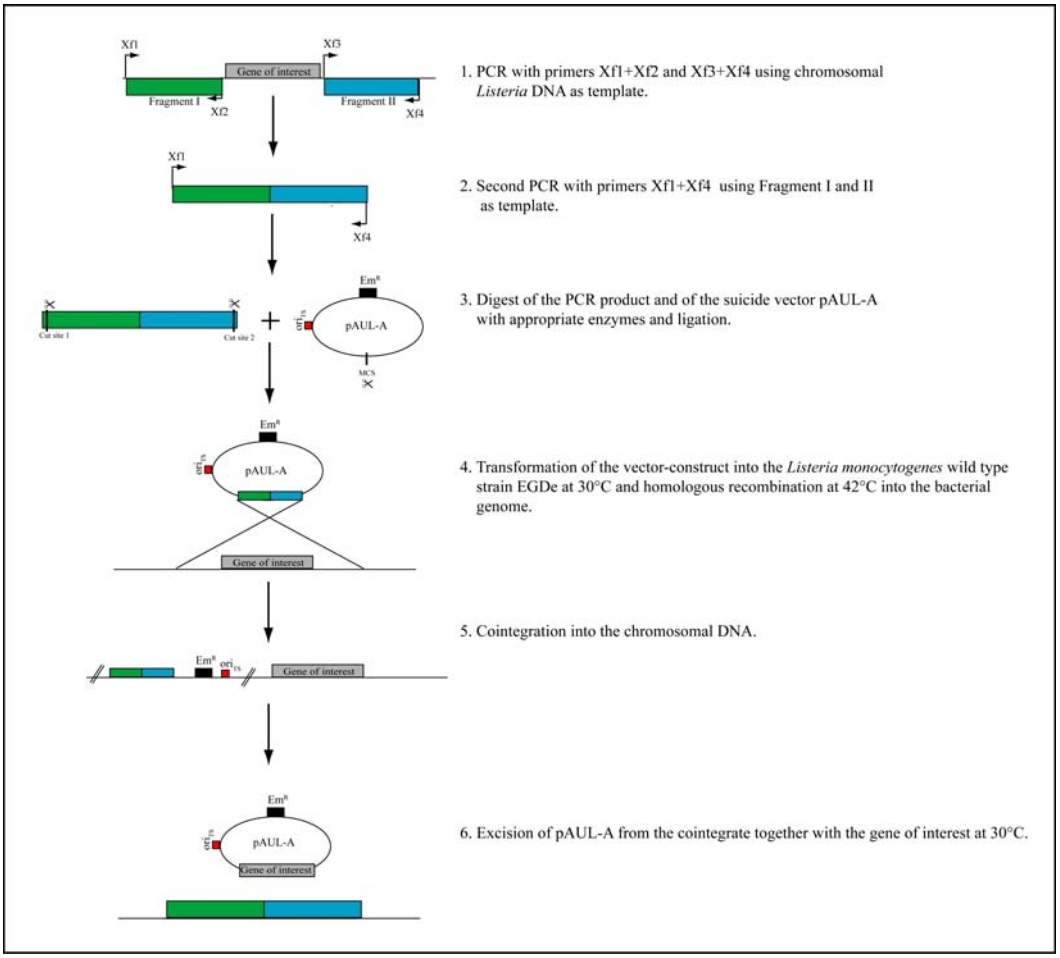
8. Appendix



Appendix A: Construction of deletion mutants in *Listeria monocytogenes* EGDe.

Hiermit erkläre ich, dass ich die vorgelegte Dissertation mit dem Titel „Molecular investigations of peptidoglycan binding proteins in *Listeria monocytogenes*" selbständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt habe, die in der Dissertation angegeben sind. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig Universität Gießen zur Sicherung guter wissenschaftlicher Praxis" niedergelegt sind, eingehalten.

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Riesengeduld viele Fragen gelöst hat und so der Arbeit immer eine Richtung gegeben hat. Mit seiner ständigen Unterstützung und seinen arbeitsaufwendigen Korrekturen der Manuskripte hat er einen entscheidenden Beitrag zum Gelingen der Arbeit geleistet.

Curriculum vitae

Persönliche Daten

Name*:*	Silke Machata
Geburtstag:	03.06.1978
Geburtsort:	Frankfurt /Main
Familienstand:	ledig
Nationalität:	deutsch

Schulische Ausbildung

1984-1989	Volksschule Germering
1989-1995	Pestalozzi- Gymnasium München
1995-1997	Spitzweg- Gymnasium Germering, Abschluss Abitur

Praktische Tätigkeit

1997-1998	Freiwilliges Soziales Jahr, Uniklinik Erlangen

Hochschulausbildung

1998-2004	Studium der Biologie für Diplom, Abschluss Diplom (mit Auszeichnung)
2001-2002	2 Auslandssemester an der Universität Albany, NY
05/2003-02/2004	Diplomarbeit am Institut für Mediziniche Mikrobiologie, Justus-Liebig-Universität Giessen: Untersuchungen zu zwei Autolysinen in *Listeria monocytogenes*
03/2004-04/2008	Promotion am Institut für Mediziniche Mikrobiologie, Justus-Liebig-Universität Giessen