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Inactivation of Lgt allows systematic characterization of lipoproteins from
Listeria monocytogenes
Inactivation of Lgt allows systematic characterization of lipoproteins from *Listeria monocytogenes*

**Running title:** Lgt of *Listeria monocytogenes*

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ABSTRACT

Lipoprotein anchoring in bacteria is mediated by the prolipoprotein diacylglyceryl transferase (Lgt), which catalyses the transfer of a diacylglyceryl moiety to the prospective N-terminal cysteine of the mature lipoprotein. Deletion of the \textit{lgt} gene in the Gram-positive pathogen \textit{Listeria monocytogenes} (i) impairs intracellular growth of the bacteria in different eukaryotic cell lines and (ii) leads to an increased release of lipoproteins into the culture supernatant. Comparative extracellular proteome analyses of the EGDe wild-type and the \textit{Δlgt} mutant provide systematic insight into the relative expression of lipoproteins. Twenty-six of the 68 predicted lipoproteins were specifically released into the extracellular proteome of the \textit{Δlgt} strain and proved deletion of \textit{lgt} is an excellent approach for the experimental verification of listerial lipoproteins. Consequently, we generated \textit{Δlgt/ΔprfA} double mutants to detect lipoproteins belonging to the main virulence regulon that is controlled by PrfA. Overall we identified three lipoproteins whose extracellular levels are regulated and one that is post-translational modified by PrfA. Noteworthy, in contrast to earlier studies in \textit{E. coli} we unambiguously demonstrated that lipidation by Lgt is not a prerequisite for activity of the lipoprotein-specific signal peptidase II (Lsp) in \textit{Listeria}. 

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Introduction

Surface proteins play key roles in the interactions of bacteria with their environment. They contribute significantly to both the adaptation to environmental changes and the uptake of nutrients and can also mediate decisive functions during the infection cycle of bacterial pathogens. In the case of *Listeria monocytogenes*, a Gram-positive bacterium responsible for severe food-borne infections in humans and animals, several surface proteins with a critical role in host-pathogen interactions have been identified (50). Depending on the expression of several essential virulence factors, all under the control of the positive transcription regulator PrfA, *L. monocytogenes* is able to invade and multiply even in non-phagocytic cells. Its ability to spread directly from cell to cell enables the facultative intracellular pathogen to cross different internal mammalian barriers leading to syndromes such as meningitis, encephalitis, sepsis or spontaneous abortion.

Surface proteins are attached to the bacterial envelope by several distinct mechanisms (5, 25). Among a total of 132 predicted surface proteins of *L. monocytogenes* (36, 49), lipoproteins presented the largest group of proteins with a common surface retention motif. Bacterial lipoproteins are characterized by the presence of specific signal peptides that are usually shorter than classical signal peptides and exhibit a characteristic consensus sequence, referred to as lipobox (commonly –Leu₃-Ser/Ala₂-Ala/Gly₁-Cys₊₁-) (38, 52). Lipoproteins are anchored to the outer surface of the cell membrane by a diacylglycerol moiety, which is covalently bound to the invariant cysteine of the lipobox. The biosynthetic pathway of bacterial lipoproteins has been studied in detail using Brauns’ lipoprotein of *Escherichia coli* (33): The key step for their subcellular localization, the lipidation, is catalyzed by the prolipoprotein diacylglycerol transferase (Lgt). Lgt mediates the transfer of the diacylglycerol moiety from phosphatidylglycerol to the sulfhydryl group of the conserved cysteine. The same position is recognized by a
lipoprotein-specific signal peptidase II (Lsp, or SPase II) that cleaves the signal peptide within the lipobox (44). Analysis of the lipoprotein pathway in *E. coli* demonstrated that modification by Lgt is a prerequisite for Lsp activity, which suggests the requirement of a modified cysteine to coordinate the processes of maturation (13, 46). In Gram-negative bacteria the N-terminal cysteine is further modified by addition of an amide-linked fatty acid by lipoprotein aminoacyl transferase (Lnt). As no orthologue of this enzyme has been found in the genomes of low G+C Gram-positive bacteria this step in lipoprotein modification is obviously not conserved in all prokaryotes (22, 30).

In Gram-positive bacteria lipoproteins perform various important roles in the milieu of the cell surface (for review see (41)). They form key components in many transport systems such as ABC-transporters, but are also known to be involved in host-pathogen interactions, e.g., initiating inflammatory processes or facilitating adherence to eukaryotic cells (1, 9, 18). The genome of *L. monocytogenes* encodes 68 putative lipoproteins comprising 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 (12). Bioinformatic genome analyses have been shown to be a powerful tool for the identification and functional assignment of putative lipoproteins (39, 40). However, pattern searches or web services created for the prediction of bacterial lipoproteins either are based on relatively small datasets of experimentally verified lipoproteins (38) or focusing on analyses of lipoproteins from Gram-negative bacteria (Lipo at [http://www.bioinfo.no/tools/lipo](http://www.bioinfo.no/tools/lipo) or LipoP at [http://www.cbs.dtu.dk/services/LipoP](http://www.cbs.dtu.dk/services/LipoP)), though lipoboxes of Gram-negative and Gram-positive bacteria seem to be similar (43). Furthermore, the influence of the process of lipoprotein maturation on their activity at the cell surface remains incompletely characterized and makes their functional investigation a
challenging task especially in Gram-positive bacteria where their expression is additionally
masked under a thick peptidoglycan layer.

Reglier-Poupet et al. (31), who inactivated the gene encoding the lipoprotein-specific signal
peptidase II of *L. monocytogenes*, already demonstrated that maturation of lipoproteins is crucial
for efficient phagosomal escape of the pathogen. However, this approach probably can not
provide information regarding the relevance of individual members of this protein family.
Inactivation of proteolytic lipoprotein processing might result in a surface accumulation of
unprocessed proteins, which would affect bacterial growth rates and their capacity to survive
hostile environmental conditions.

Here we report the effects of inactivation of the second enzyme involved in the lipoprotein path-
way of *L. monocytogenes*, the prolipoprotein diacylglyceryl transferase (Lgt). We precisely de-
finied its role for retention and translocation in comparison to Lsp in *Listeria* and in addition
present evidence that casts new light on dogmas of bacterial lipoprotein processing. Noteworthy,
deletion of *lgt* did not affect the secretion of any non-lipoprotein in *Listeria*. Exclusively lipo-
proteins were released in addition to normally secreted proteins into the extracellular
compartment and their expression could be analyzed systematically. To detect lipoproteins
probably involved in the pathogenesis of *L. monocytogenes* and responsible for decreased
intracellular replication of a *lgt* deletion strain we applied this strategy to detect those that are
regulated by the virulence gene regulator PrfA. Proteome analyses of differently PrfA-expressing
Δ*lgt* strains revealed several regulated lipoproteins and indicate a PrfA-dependent post-
translational modification of the oligopeptide-binding protein (OppA).
MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

*Listeria* strains used in this work are the *L. monocytogenes* EGDe wild-type (12), and the isogenic strain Δ*prfA* (8). The Δ*lgt* strain, the complemented Δ*lgt* (pE1lgt) strain, the Δ*lgt* (pES11) strain, the Δ*lgt*/Δ*prfA* (pES11) strain and the PrfA-overexpressing Δ*lgt* (pEPS11prfa) strain were generated in the same genetic background (this work).

Strains were grown in Brain-heart infusion (BHI) or minimal medium, supplemented with 5µg/ml erythromycin when carrying plasmids. Cultivations of bacteria in minimal medium were performed as previously described (49). For the inactivation of lipoprotein-specific signal peptidase II (Lsp) 20mg Globomycin (Sankyo Co., Tokyo) was dissolved in 1ml 70% (v/v) ethanol and applied to BHI at a concentration of 100µg/ml.

Construction of the expression vectors pE1, pES11 and pEPS11

To obtain the pE1 expression vector the pAT28 vector (48) was digested with XbaI and SphI. A new polylinker region (with restriction sites for NdeI, NcoI, NruI, ClaI, BglII, SalI, XbaI) was inserted using the oligonucleotides: 5’-TCGAGCATATGCCATGGTCGCGAATCGATA GATCTGTCGACTGAGTAGGTAAT-3’ and 5’-CTAGATTACCTACTCAGTCGACAGATC TATCGATTCGCGACCATGGCATGCTCGAGCATG-3’. The Linker was annealed and phosphorylated and generated XbaI and SphI overlaps. It was cloned into the digested pAT28 vector. The resulting derivative (pLig46) was digested with NdeI and XhoI. A 0.23 kb fragment encompassing the promoter region of actA was PCR amplified using *L. monocytogenes* total DNA as template and the primer pair: 5’-TTTAATCCCATATGCTACTCCCTCCTCAGC-3’ and 5’-TGAAGCTCGAGAAGCATGTTGGG-3’ containing the sequence recognized by restriction
endonuclease NdeI and XhoI. The amplification product was digested with NdeI and XhoI and ligated to pLig46. This generated the derivative (named pLig57) which was used for integration of a part of the \textit{lisA} terminator region. For this reason pLig 57 was digested with XbaI and cloned to a 85bp fragment encompassing the terminator region of \textit{lisA} which was amplified using \textit{L. monocytogenes} total DNA and the primer pair: 5’-AATAAAAAATCTAGAATATAAAACCGCTTAAC-3’ and 5’-GATAAACATCTAGATATTCTTTTACATTTTG-3’ containing a sequence recognized by restriction endonuclease XbaI at the 5’- and 3-end of the fragment. The fragment was digested with XbaI and cloned into pLig 57. The resulting recombinant plasmid pLig57b was necessary as cloning vector for the final expression vector pE1. To obtain pE1 the pLig57b was digested with XhoI and BamHI, the resulting 0.5 kb-fragment was cloned into the vector pCGU34 (27) digested with SalI and BamHI. For generation of pES11 the following primers were used for PCR amplification using \textit{L. monocytogenes} total DNA as template to amplify the promoter region and the signal sequence of \textit{actA}: 3’-CTAGAATCTTCCATATGTGCTGCTGCAAA-3’ and 5’-TGAAGCTCGAGAAGCAGTTGGGT-3’ (restriction sites underlined). The 0.35kb fragment was digested with NdeI and XhoI analogue to the cloning strategy described above (using pLig57b as cloning vector). Generating a vector (pLig76) with preferred \textit{Listeria} codon usage: 5’-CAT.ATG.CCA.TGG.TCG.CGA.ATC.GAT.AGA.TCT.GTC.GAC.TGA.GTA.GGT.AAT.CTA.GAA -3’ (reading frame indicated by dots) of actA. The XhoI/BamHI fragment (approximately 0.5 kp) of this vector was transferred to pCGU34 digested with BamHI and SalI to generate the pES11. To obtain pEPS11 the pES11 was cut with KpnI and SacI. A 1 kb fragment encompassing the prfA gene was PCR amplified using \textit{L. monocytogenes} total DNA as template and the primer pair 5’-AGCAACCTCGGTACCATAT-3’ and 5’-CTGTTGGAGCTCCTTCTTGGTGAAGCAATCG-3’ containing the sequence recognized by restriction endonucleases KpnI and SacI (underlined).
amplification product was digested and cloned into the KpnI and SacI sites of pES11, thereby generating pEPS11.

**Generation and complementation of the Δlgt deletion mutant**

The flanking regions of the *lgt* gene were amplified by PCR from the chromosome of *L. monocytogenes* EGDe wild-type. The primer pair Lgt1-F (CGTTGATTGGTTCTCTCCGGCG) and Lgt2-R(Xba1) (GAACACCATTATCTAGAATTCCTAC) was designed to amplify a 459bp fragment at the 5'-flanking region of the *lgt* gene. The primer pair Lgt3-F (Xba 1) (GGTAAAGTAGTCTAGAGAATAAAAAAGTTGG) and Lgt4-R (ATCTCTTCA TATAAAGCACAATCGC) was used to amplify a 358bp fragment at the 3'-flanking region of the *lgt* gene. After restriction with Xba1, the two fragments were ligated and cloned into the pCR-blunt 11-Topo vector using the TA Cloning™ kit (Invitrogen, Karlsruhe, Germany). The plasmid was isolated from the recombinants, digested with HindIII and EcoRV and the inserted DNA was cloned into the HindII/Smal-restricted pAULA vector. Following duplication of pAUL-A-Δlgt in *E. coli* TG2, pAUL-A-Δlgt was electroporated into *L. monocytogenes* EGDe wild-type and gene replacement was performed as previously described by Schaferkordt and Chakraborty (35). The deletion was confirmed by PCR sequence analysis of chromosomol DNA from the Δlgt mutant. For the construction of the ΔprfA/Δlgt mutant pAUL-A-Δlgt was electroporated into a *L. monocytogenes* ΔprfA mutant (8). Subsequent steps were carried out analogous to the construction of the Δlgt mutant. To complement the Δlgt mutation, *lgt* was amplified by PCR from the chromosome of the EGDe wild-type using the primer pair Lgt5-F(Ndel) (GAGCATATGATGGGTAATGGTTCAGC) and Lgt6-R(Sall) (GCGCGTCGACCTTCTTTCTTAATCAAAAC TCG). The DNA fragment was digested with Sall and Ndel and inserted into the Sall/Ndel-restricted shuttle vector pE1 downstream of the constitutive
actA promoter. After duplication of pE1lgt in E. coli TG2, PCR sequence analysis was performed to verify the sequence of the cloned gene. pE1lgt was transformed into the Δlgt strain by electro-poration and selection of transformants was performed on BHI agar plates supplemented with 5µg of erythromycin per ml.

**Generation of polyclonal antibodies and immunoblot analysis**

Polyclonal antibodies were prepared by immunization of rabbits with synthetic peptides. Peptides corresponding to residues 35-47 of the *L. monocytogenes* Lgt sequence, to residues 31-50 of the Lmo1800 sequence and to residues 73-92 of the Lmo2595 sequence were synthesized with a terminal cysteine residue by the Dept. of Chemical Biology (GBF, Braunschweig). The peptides were conjugated with a maleimide-activated carrier protein using the "Imject Maleimide Activated Immunogen Conjugation Kit" (Pierce /Perbio, Bonn, Germany) and subsequent immunizations of rabbits were performed by Biogenes GmbH (Berlin, Germany). IgGs were isolated by affinity chromatography on CNBr-activated Sepharose 4B (Amersham Biosciences, Freiburg).

Lgt expression was analyzed in protein extracts prepared from cultures of bacteria grown in BHI broth (OD<sub>600</sub>=1.8, stationary phase). Proteins were separated by SDS-PAGE (21) and blotting was performed with PVDF membranes in a semidry blot chamber as described (47) with 1 mA/cm<sup>2</sup> for 1 h. Lgt was detected with polyclonal antibodies (this work; final dilution 1:1000) and peroxidase- coupled goat anti-rabbit secondary antibodies (Dianova, Hamburg, Germany).

For analysis of Lmo1800 and Lmo2595 expression, different amounts of extracellular protein extracts were transferred to PVDF membranes using a slot blot unit. Protein detection was carried out with polyclonal antibodies (this work; final dilution 1:1000), whose specificities had previously been tested by SDS-PAGE and immunoblot analyses of total protein extracts (data not shown). Chemoluminescent detection was performed with the Lumi-Light Western Blotting
Substrate (Roche, Mannheim, Germany) and a cooled CCD camera LAS-1000 (Raytest, Straubenzahn, Germany). N-terminal sequencing was performed according to (4).

**Metabolic labeling of lipoproteins with [14C]-palmitic acid**

Bacterial strains were cultivated in 20ml of BHI medium supplemented with 10µCi [14C]-palmitic acid. Cells were harvested by centrifugation (10min, 3,000xg) at late exponential phase and washed twice with PBS. For generation of protoplasts, pelleted cells were resuspended in 2.5ml of protoplast buffer (1 M sucrose, 30mM NaCl, 10mM MgCl x 6H2O, 50mM Tris-HCl, pH7.5) containing 0.33mg/ml of bacteriophage endolysin Hpl511 (23) and 0.01 mg/ml mutanolysin (Sigma-Aldrich, Taufkirchen, Germany). After incubation for 10 min at 37°C, 2.5ml of MQ water and a protease inhibitor (Complete™, Roche, Mannheim, Germany) were added. Protoplasts were disrupted by incubation on ice (10min) followed by homogenization with a Potter glass homogenizer. After centrifugation at 10,000xg (5min, 4°C) to remove cell debris and remaining bacteria, membranes of the supernatant were pelleted by centrifugation at 100,000xg for 20min (4°C). Isolation of membrane proteins was performed by chloroform/methanol extraction as previously described by (53). Proteins were separated by SDS-PAGE, transferred to a PVDF membrane and exposed to a Fujifilm imaging plate (type BAS-MS 2025) for 2-3 weeks. [14C]-labeled protein bands were visualized with a Fujifilm BAS-2500 phosphorimager (Raytest, Straubenzahn, Germany).

**Preparation and 2D-PAGE of extracellular protein fractions**

500 ml cultures of bacteria cultivated in ultrafiltrated (10kDa cutoff) BHI or minimal medium were harvested by centrifugation (3,000xg, 10 min, 4°C). The culture supernatants were filtered (0.22µm) and extracellular proteins were precipitated as previously described (49). The dried
protein pellets were resolved in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30mM DTT, 1.65 mM Tris 0.5% IPG buffer (Amersham Biociences), protease inhibitor (Complete™, Roche) and protein concentration was determined according to Bradford (1976). Isoelectric focussing was performed on IPGphor units using 18 cm immobilized pH gradients (pH 4-7) at a total voltage of 100-120kVh (0V for 4h, 30V for 10h, 30-300V for 3h, 300-3,000V for 6h, 300-5,500V for 3h, 5,500V till the end). The second dimension was carried out as described (36) using 12-15% gradient polyacrylamide gels. The gels were stained with RuBPS according to Rabilloud (29), recorded (CCD camera LAS-1000, Raytest, Straubenhardt, Germany) and subsequently analyzed with ProteomWeaver 2.1 (Definiens, Munich, Germany).

**Protein identification by mass spectrometry**

For mass spectrometry, proteins cut from the 2D gels were handled as described by (36). Proteins were identified by peptide-mass fingerprint as well as post-source decay fragmentation data recorded on a Bruker Ultraflex MALDI-TOF mass spectrometer (Ultraflex, Bruker Daltonics, Bremen). Evaluation of the spectra was carried out with the Biotools software (Bruker Daltonics) along with the MASCOT search engine (version 1.9; Matrix Science, London, UK). The criteria used to accept protein identifications included the extend of sequence coverage (minimum of 30%), the number of peptides matched (minimum of 5) and the score of probability (minimum of 70 for the Mowse score). Lower-scoring proteins were either verified manually or rejected.

**In vitro infection analyses**

The mouse fibroblast cell line 3T3 (ACC 173, DSMZ) and the human epithelial cell line Caco-2 (ACC 169, DSMZ) were cultured in Dulbecco's modified Eagle medium (DMEM, 1,000mg/ml glucose; Gibco BRL / Invitrogen, Karlsruhe, Germany) supplemented with 2 mM L-glutamine
and 10% fetal calf serum (FCS) at 37°C in the presence of 7% CO₂. For the infection analysis, cells were cultivated to a density of 6x10⁴ cells/cm² in 6-well tissue culture plates (Nunc) or for microscopic studies; onto 3x12mm glass coverslips in 6 well plates. Monolayers were used for infection analyses 24h (3T3) or 48h (Caco-2) after seeding. Bacteria from 14h cultures in BHI (stationary phase) were pelleted by centrifugation, washed once with PBS and diluted in DMEM supplemented with 2mM L-glutamine. Before inoculating cells with bacteria at MOI’s of approximately 6 or 100 bacteria per cell for microscopic studies, respectively, cells were washed twice with PBS and overlaid with DMEM containing 2mM L-glutamine but lacking FCS. Bacteria were centrifuged onto cells at 1,000xg for 4 min. After 30 min of incubation, infected cells were washed twice with PBS and overlaid with fresh DMEM supplemented with 2mM L-glutamine, 10% FCS and 50µg/ml Gentamycin to kill extracellular bacteria. At selected intervals (1, 3, 5, and 7h), cells were washed six times with PBS and lysed by addition of ice-cold 0.2% Triton X-100 for 1 min. Viable bacteria released from the cells were plated onto BHI agar plates to count colonies after one day. Each assay was carried out in triplicate and repeated three times. Double fluorescence labeling of F-actin and Listeria was performed as described previously by (8) using phalloidin coupled to Alexa Fluor™ 488 (Molecular Probes / Invitrogen, Karlsruhe, Germany) and polyclonal antibodies against L. monocytogenes revealed with Alexa Fluor™ 594-conjugated goat anti-rabbit (Molecular Probes). Samples were mounted and viewed with an epifluorescence microscope (Axiovert 135TV, Zeiss, Göttingen, Germany).

**Bioinformatic lipoprotein analysis**

A Hidden Markov model was built based on the available 26 verified lipoproteins. The multiple alignment was created with ClustalW using the shortest N-terminal sequence set – 31 amino acids - that yielded an alignment with all essential cysteines lined up. The HMM was created
using hmmbuild (HMMER package, 2.3.2 release, available at http://hmmmer.wustl.edu). The complete genomic protein sequences of *L. monocytogenes* EGDe, *L. innocua* (12) and *L. monocytogenes* F2365 (26) downloaded from GenBank (AL592022.faa, AL591824.faa, AE017262.faa) were then extracted with a BioPerl script to produce a FASTA-formatted file with the N-terminal 40 amino acids of only those proteins that feature at least one cysteine between position 15 and 30 of their sequence to remove sequences that cannot be lipoproteins (the cysteine of the verified lipoproteins is at position 18 to 23). These files were then analyzed by hmmsearch with the cutoff E-value set to 100.
RESULTS

Deletion of \textit{lgt} does not affect bacterial growth in BHI but impairs intracellular growth within host cells

The open reading frame \textit{lmo2482} in the genome of \textit{L. monocytogenes} EDGe wild-type has been annotated as prolipoprotein diacylglycerol transferase (\textit{lgt}) (12). \textit{lmo2482} encodes a protein of 277 amino acids with a calculated molecular mass of 31.7 kDa. The gene product contains seven putative transmembrane domains (3) and has 59\% amino acid identity with Lgt from \textit{B. subtilis}. A homology search by Blast revealed that Lgt is highly conserved in the genus \textit{Listeria}, sharing 98-100\% identity with the published \textit{L. monocytogenes} genomes of the food isolates F2365, F6854 and H7858 and 94\% amino acid identity with Lgt (Lin2625) of the non-pathogenic species \textit{L. innocua}. As observed for other bacteria no further open reading frame coding for a prolipoprotein diacylglycerol transferase paralogue could be deduced from these genome analyses. We inactivated the proposed \textit{lgt} gene of \textit{L. monocytogenes} EDGe by constructing an in-frame deletion mutant. Compared to the parent strain the mutant (\textit{Δlgt}) displayed no differences with respect to cell and colony morphology and \textit{in vitro} growth analyses that were performed in brain-heart infusion (BHI) at 37°C (data not shown). The growth rates of both strains were almost identical in the logarithmic phase. This observation indicated that Lgt is not essential for viability or cell division in rich medium.

Next we characterized the \textit{Δlgt} strain (i) under nutrient stress and (ii) more hostile conditions occurring during the process of host cell invasion and intracellular growth. Cultivation in minimal medium resulted in slightly lower growth rates and a reduced final optical density of the deletion strain compared to the wt (Figure S3). To analyze whether lipoproteins and in particular their lipidation contribute significantly to the pathogenicity of \textit{L. monocytogenes}, we exposed the
non-professional phagocytic mouse fibroblast cell line 3T3 to the wild-type strain or the Δlgt mutant using a multiplicity of infection of 100 (bacteria / cell). Three hours post infection double staining with an anti-Listeria antibody and β–phalloidin to visualize F-actin showed that the Δlgt mutant was generally able to invade and to replicate within the mouse fibroblast cell line. The mutant strain also formed actin tails similar to the wild-type, indicating that inactivation of lgt did not interfere with intracellular motility (Figure 1A).

To analyze in detail which effects the lgt deletion had on invasion or intracellular survival of Listeria, we performed further experiments with the mouse fibroblast cell line 3T3 and the human epithelial cell line CaCo-2. Both cell lines were exposed for 30 min to the wild-type strain or the Δlgt mutant using a multiplicity of infection of 6. Cells were lysed after various intervals (1, 3, 5 and 7h post infection) and the number of intracellular bacteria was determined by counting colonies plated on BHI agar. No significant difference (P>0.01; Student’s test) between the wild-type and the Δlgt mutant was observed 1h post infection in both cell lines (Figure 1B). This observation indicated that lgt inactivation has no effect on bacterial entry into these cell lines. In contrast, 3, 5 and 7h post infection the number of intracellular Δlgt bacteria was significantly lower than those of the wild-type. Both cell-lines revealed maximal relative reduction levels three hours post infection which was about 3-fold and 2-fold for 3T3 and Caco-2 cells, respectively. At later time points we observed lower but still significant relative differences between wt and deletion mutant. In conclusion the in vitro infection results demonstrated that prolipoprotein anchoring by Lgt contributes to intracellular growth of L. monocytogenes.

**Lgt is exclusively responsible for lipidation of prolipoproteins in L. monocytogenes**

To ascertain that the effects described above are exclusively mediated by Lgt acting on lipoproteins we established a complementation strain by transforming the Δlgt mutant with a multicopy
plasmid harboring the wild-type *lgt* gene downstream of a constitutive promoter. The resulting strain is referred to as Δ*lgt*(pE1lgt). To analyze the expression of Lgt within the wild-type and the complemented strain we generated polyclonal antibodies. Immunoblot analysis using the affinity-purified anti-Lgt antibodies detected an increased expression of Lgt within the Δ*lgt*(pE1lgt) strain compared to the wild-type. As expected, Lgt expression was abolished in the Δ*lgt* mutant strain (Figure 2A).

To determine whether the absence of Lgt also coincides with a completely abolished protein lipidation we carried out metabolic labeling experiments with [14C]-palmitic acid. The Δ*lgt* mutant, the wild-type and the Δ*lgt*(pE1lgt) strain were cultivated in BHI in the presence of [14C]-palmitic acid and harvested at late exponential phase. To ascertain the specificity of the labeling we first generated and purified protoplasts and then isolated membranes for the comparative analysis. Autoradiography of lipoprotein extracts separated by a SDS-PAGE revealed several [14C]-labeled protein bands in the samples of the wild-type strain and the complemented mutant (Figure 2B). Although the level of Lgt expression was found to be increased in the complemented strain, patterns and relative abundances of labeled proteins were remarkably similar compared to the wild-type strain. In contrast, no isotopically labeled protein was detected in the Δ*lgt* strain, demonstrating that Lgt is exclusively responsible for the lipid modification of lipoproteins.

**Deletion of lgt facilitates systematic identification and characterization of lipoproteins**

To investigate the effects of *lgt* inactivation on lipoprotein anchoring in *L. monocytogenes* we examined the extracellular proteome of the Δ*lgt* mutant by 2D gel electrophoresis. Bacteria were cultivated in BHI and proteins of the supernatant were obtained as previously described by Trost et al. (49). We chose a pH gradient from 4 to 7 for the first gel dimensions, as only five of the 68 annotated lipoproteins (Lmo1903, Lmo2349, Lmo0821, Lmo1379, Lmo2854) have calculated
isoelectric points above 7 (Supplementary data, Table S1). The extracellular expression pattern of the $\Delta lgt$ mutant displayed striking differences to that of the wild-type (Figure 3A). Several spots were detected exclusively or significantly upregulated in the extracellular proteome of the $\Delta lgt$ mutant. Analyses of these spots by mass spectrometry resulted in the identification of 24 different proteins. All of these belong to the group of putative lipoproteins, including various substrate-binding proteins, proteins involved in specific enzymatic activities as well as several proteins of unknown function (Table 1). The apparent pI and molecular mass values of the majority of lipoproteins detected in the extracellular proteome of the $\Delta lgt$ mutant correlate extremely well with their theoretical values calculated without their signal peptides. This observation suggested that signal peptides were cleaved off specifically and lipoproteins were released without further post-translational modifications after inactivation of $lgt$. One exception was the lipoprotein Lmo2219 (spot 9, Figure 3A) that was detected in two different spots, featuring slight differences in their pIs and molecular masses.

OppA and Lmo0135 (spot 1 and 2, Figure 3A) were detected in the 2D gel of the wild-type in amounts comparable to the other resolved proteins, but dominate the extracellular proteome of the $\Delta lgt$ mutant. Both are annotated as oligopeptide-binding proteins associated with ABC-type transport systems and therefore indicate a particular role of oligopeptide-transport for maintaining the physiological state of bacteria in rich media. Further two annotated di/oligopeptide-binding proteins were identified (Lmo0153, Lmo2569) but their expression was low compared to Lmo0135 and OppA.

Whereas inactivation of Lgt from B. subtilis resulted in the release of different protein species into the supernatant (2), no additional or significantly increased protein species besides the annotated lipoproteins could be detected in the protein pattern of the L. monocytogenes $\Delta lgt$ mutant compared to the wild-type. Though our observations suggest inactivation of $lgt$ as an excellent
strategy for the systematic investigation of listerial lipoproteins, we found one lipoprotein, QoxA, whose surface retention was not impaired by the Δlgt mutation. QoxA was detected in comparable amounts in the extracellular proteome of the wild-type and the Δlgt mutant, however, only in a spot whose apparent molecular mass was about 10kDa lower than the theoretical value (“QoxA”, Figure 3A).

Comparative analysis of the extracellular proteome of the wild-type and the complemented strain demonstrated that expression of Lgt in the Δlgt mutant restored lipoprotein anchoring. Note-worthy, no significant differences in the extracellular expression pattern were observed for both the lipoproteins and the other extracellular proteins (Figure 3B). Even lipoproteins such as OppA and Lmo0135, which could always be found as part of the culture supernatant of the wild-type, were detected in comparable amounts in the growth medium of the complemented strain. This analysis demonstrated that overexpressing Lgt has no detectable effect on the release of lipoproteins and other secreted proteins thus underlining the specificity of its enzymatic function.

**Retention but not translocation of listerial lipoproteins depends on Lgt**

To investigate the process of lipoprotein release in the Δlgt mutant we performed N-terminal sequencing of the eight most abundant validated lipoproteins and the putative QoxA-fragment. We found that QoxA exhibits two N-terminal transmembrane domains. The extracellular QoxA spot was cleaved immediately behind this region. As shown in *Bacillus subtilis* (2) shedding of QoxA into the supernatant occurs independently of lipidation by Lgt. Individual N-terminal sequences could not be determined for OppA and Lmo0135, however all other lipoproteins unambiguously revealed N-terminal sequences in perfect accordance with the cleavage site predictions of Lsp (Table 2). Only Lmo2219 was processed in two slightly different forms. Whereas the acidic form (see spot 9, Figure 3A) represents the perfect mature lipoprotein, the basic form is a homogenous
fraction of Lmo2219 starting with alanine at position -1. Apart from this peculiarity Lsp seemed to be exclusively responsible for the processing of lipoproteins. Furthermore, the lipidation by Lgt, which ensures the retention at the outer surface of the membrane, is obviously not an essential prerequisite for the Lsp-dependent processing of listerial prolipoproteins. This observation was surprising since previous studies on other species concluded that Lsp can only act on diacyl-glyceryl modified prolipoproteins (7, 13, 45).

To prove that Lsp is actually responsible for the processing of prolipoproteins in *L. monocytogenes* we cultivated the Δlgt strain in the presence of Globomycin which specifically inhibits lipoprotein-specific signal peptidase II activity (7, 16, 34). The release of the majority of lipoproteins into the supernatant was now much impaired resulting in a proteome pattern of the Δlgt strain looking remarkably similar to that of the wild-type (Figure 4). Only Lmo0135 and Lmo2219 (basic form, starting with alanine) were still released into the supernatant. A comparison between these proteins with all other lipoproteins that were retained in the presence of Globomycin did not indicate a biochemical property that would explain the different retention behavior. Thus, the majority of prolipoproteins most likely can not complete their translocation across the membrane if Lsp is inhibited. In conclusion, Lgt activity is responsible for membrane retention, but is no prerequisite for signal peptide processing and translocation of listerial lipoproteins.

**Identification of PrfA-regulated lipoproteins**

Different virulence factors of *L. monocytogenes* can be naturally detected as part of the extracellular proteome (49) and are under the positive control of the main regulator of virulence PrfA. Deletion of *lgt* affords access to lipoproteins for expression studies within this subproteome, permitting the identification of those which may play a significant role during infection. Thus, we
constructed Δlgt strains with different PrfA expression and compared the lipoprotein expression profiles of these strains by 2D gel electrophoresis. A ΔprfA/Δlgt strain was obtained analogous to the Δlgt strain and a constitutive PrfA-overexpressing Δlgt strain was constructed by transforming the Δlgt mutant with a multicopy plasmid harbouring the prfA gene of the L. monocytogenes strain NCTC 7973 downstream of a constitutive promoter (Δlgt (pEPS11prfA)). Since additional effects on the extracellular proteome caused by this multicopy plasmid alone could not be excluded we also transformed the Δlgt strain and the ΔprfA/Δlgt strain with the empty vector. The resulting strains are termed ΔprfA/Δlgt (pES11) and Δlgt (pES11). Bacteria were cultivated in minimal medium, since the expression of PrfA is generally reduced in a rich medium such as BHI (24). Growth analyses in minimal medium at 37°C revealed a slight growth advantage of the ΔprfA/Δlgt (pES11) strain over the Δlgt (pES11) strain that corresponds most likely to the difference in the transcription of virulence factors under PrfA-induced conditions. Consequently, growth of the constitutively PrfA expressing Δlgt (pEPS11prfA) strain was even further delayed compared to the other strains (Figure 5). Hence we harvested bacteria of both the ΔprfA/Δlgt (pES11) and the Δlgt (pES11) strain at different time points but always in the late exponential growth phase. Bacteria of the PrfA-overexpressing Δlgt strain were harvested isochronal to the Δlgt (pES11) at an OD600nm of 0.4 and its supernatant exhibited total protein content very similar to that of both other strains. Protein secretion that is the prerequisite for the activity of many virulence factors is obviously markedly increased by overexpression of PrfA. Comparative relative expression analyses of the extracellular proteomes by 2D gel electrophoresis revealed several spots which were exclusively detectable or significantly increased in the extracellular proteome of the Δlgt (pES11) strain compared to that of the ΔprfA/Δlgt (pES11) strain. The abundance of these proteins was further increased in the extracellular proteome of the
Δlgt (pEPS11prfA) strain, demonstrating a direct positive correlation with the amount of PrfA (Figure 6). All regulated spots were analyzed by MALDI-TOF mass spectrometry and resulted in the identification of six different proteins. Three of these proteins were identified as the well-known PrfA-regulated proteins Internalin (InlA), Internalin C (InlC) and the actin-assembly inducing protein ActA. The other proteins were identified as the predicted lipoprotein Lmo0366 that was exclusively detected in the PrfA-overexpressing strain, Lmo2219, and the oligopeptide-binding protein OppA(1). Lmo2219 shares 45% identity with the post-translocation chaperone PrsA of *B. subtilis*.

One abundant protein was detected in the Δlgt/ΔprfA (pES11) mutant but its expression was reduced by concurrent expression of PrfA as demonstrated in the Δlgt (pES11) and the Δlgt (pEPS11prfA) strains. Mass spectrometric analysis unambiguously identified this spot also as the oligopeptide-binding protein OppA. Its apparent isoelectric point differs markedly from that of the other OppA spot which was in agreement with the calculated pI of 5.26 (without signal sequence), thus we termed it OppA(2). Since its apparent MW is identical to OppA(1) the observed pI shift strongly suggest a PrfA-regulated post-translational modification of OppA. No PrfA-dependent alteration of the total OppA protein level (OppA(1)+OppA(2)) could be detected.

To complement this comprehensive search for PrfA-regulated lipoproteins we extended our analysis to low abundant lipoproteins that could not be detected on the 2D-gels but may be involved in the pathogenic lifestyle of *L. monocytogenes*. We generated polyclonal antibodies against the predicted lipoproteins Lmo1800 and Lmo2595, that attracted our attention because Lmo1800 is a putative tyrosine phosphatase, an activity associated with virulence of YopA of *Yersinia* (10). Lmo2595 is a protein of unknown function which has no orthologue in the closely related apathogenic species *L. innocua*. Surface anchoring of both predicted lipoproteins was significantly impaired by inactivation of *lgt* (Figure 7A) raising the number of experimentally
validated lipoproteins to 26. To analyze whether these proteins are regulated by PrfA we compared the extracellular proteomes of the different PrfA-expressing Δlgt strains by immunoblot analyses. Whereas Lmo1800 showed no PrfA-dependent expression, the extracellular amount of Lmo2595 was significantly decreased in all PrfA-expressing Δlgt strains, suggesting a negative regulation of Lmo2595 by PrfA (Figure 7B).

A Hidden Markow Model (HMM) for listerial lipoprotein prediction

Besides the detection of 4 PrfA-regulated lipoproteins a total of 26 proteins were experimentally verified as lipoproteins in this study (Table 1) that can be used to improve the quality of predictive tools. Thus, we created a new Hidden Markow Model for lipoprotein prediction in the genus *Listeria*. Analysis with hmmsearch yielded 62 hits in *L. monocytogenes* EGDe (Supplementary data, Table S2). This set included all verified lipoproteins among the first 36 ranked proteins. Except for one candidate (Lmo1340) all predicted lipoproteins agree with the former annotation of Glaser et al. (12). The lipobox of Lmo1340 (Ser-3-Phe-2-Gly-1-Cys+1) differs only slightly from the consensus sequence (Leu-3-Ser/Ala-2-Ala/Gly-1-Cys+1-) and thus we assume that Lmo1340 is lipidated by Lgt. Following the criteria of Sutcliffe and Harrington (38) for “false-positives” we examined the signal sequences of the seven putative lipoproteins which were annotated by Glaser et al. (12) but not detected with the HMM. Among these we found the predicted lipoprotein Lmo0810 as a “false-positive”. The putative Lipobox of Lmo0810 does not contain a cysteine at position +1 of the cleavage site and thus lipid modification of Lmo0810 by Lgt can be excluded.

Searching the *L. innocua* genome produced an analogous result of 61 hits, removing Lin0800, the orthologue of Lmo0810, and Lin0626 with the cysteine at position 13 from the previous prediction. In addition, Lin1764 – unique to *L. innocua* – was predicted as a further lipoprotein.
The genome of *L. monocytogenes* F2365 was predicted to contain 70 lipoproteins ((26), Table S4, number given but proteins not listed), However, only 21 are annotated as such (UniProt) and a further 5 have an InterPro entry indicating lipid modification. Our HMM analysis identified 56 proteins as putative lipoproteins (Supplementary data, Table S2). Three further proteins annotated as “lipoprotein, putative” in this genome were predicted only with low significance. One of these, LMOf2365_2112 might well be a true lipoprotein with a divergent lipobox. In the case of LMOf2365_0173 and LMOf2365_2187 we regard the original annotation as probably incorrect (Supplementary data, Figure S2).
DISCUSSION

In this study we confirmed that the open reading frame lmo2482 of *L. monocytogenes* EGDe annotated as prolipoprotein diacylglycerol transferase (*lgt*) (12) is solely responsible for the lipid modification of proteins. Deletion of the *lgt* gene completely abolished metabolic labeling of proteins with $[^{14}\text{C}]$-palmitic acid and also led to a prominent release of lipoproteins into the extracellular milieu, indicating that lipidation is not required for Lsp-dependent signal peptide processing and translocation across the membrane.

Lipoprotein generation by Lgt seems to be essential for growth in the Gram-negative bacteria *Escherichia coli* and *Salmonella typhimurium* (11, 33). In striking contrast, inactivation of the *lgt* gene does not affect viability in *Bacillus subtilis*, *Streptococcus pneumoniae* and *Staphylococcus aureus* (22, 28, 37). It is conceivable that in contrast to Gram-negative bacteria unmodified lipoproteins of Gram-positive bacteria retain their biological activity at least partially or are involved in less important functions. This hypothesis is supported by investigations of the lipoprotein PrsA in *B. subtilis*, an extracellular chaperone which mediates stability of exported proteins and thus plays an important role in protein secretion (17). Deletion of the *prsA* gene is lethal, indicating that PrsA is essential for growth in *B. subtilis* (20, 22). The *lgt* knock-out of *B. subtilis* is, however, fully viable, but significantly impaired in protein secretion (22). Whereas inactivation of *lgt* had no visible effect on *in vitro* growth in rich medium, it clearly influenced intracytosolic growth of *L. monocytogenes*. This corroborates the importance of the results of our *in vitro* infection analyses, which demonstrated that selecting the correct growth conditions is pivotal for recognizing the real significance of gene functions. The Δ*lgt* mutant did not affect invasion into non-phagocytic cells, but always showed a significantly reduced multiplication within the different cell lines. The fact that relative differences between wt and deletion mutant are
maximal 3 hour post infection but not at later time points might indicate either a specific role of lipoproteins in the course of infection or compensatory capacities of *Listeria* that warrant further studies.

Besides lipoprotein anchoring maturation of prolipoproteins by the lipoprotein-specific signal peptidase II (Lsp) has also been demonstrated to affect intracellular survival of *L. monocytogenes* (31). Inactivation of the *lsp* gene resulted in a severe growth defect of *L. monocytogenes* in BM macrophages, which was accompanied by a reduced capacity to escape from phagosomal compartments. Inactivation of *lsp* in *Mycobacterium tuberculosis* also leads to a markedly reduced virulence of this human pathogen (32) and deletion of *lgp* in *Streptococcus pneumoniae* resulted in decreased growth of *S. pneumoniae* in the respiratory tract of infected mice (28). As demonstrated by these examples, interfering with the lipoprotein pathway represents a powerful strategy for analyzing the role of lipoproteins in the infectious process of different bacteria. However, it provides no information on whether the alteration of a single lipoprotein or the cumulative effects of changes in the biological activity of numerous lipoproteins are responsible for the attenuated phenotype. In order to answer this question it is necessary to examine first which proteins are really post-translational lipid modified and thus directly affected by the targeted mutation.

**Experimental and predictive characterization of listerial lipoproteins**

An increased release of lipoproteins into the extracellular proteome of a Δlgp mutant has previously been reported in *B. subtilis* (2). However, the extracellular levels of some autolysins were also increased in this mutant, demonstrating that inactivation of *lgp* also has an indirect effect on the release of non-lipoproteins. In contrast, we found no difference in secretion of non-lipoproteins between the Δlgp mutant and the wild-type of *L. monocytogenes*, although we improved our comparative proteome analysis by the use of narrow pH-gradients in the first gel dimension.
(Supplementary data, Figure S1). These data demonstrated our comparative extracellular proteome analysis as a simple and very reliable approach for the experimental verification of protein lipidation. Overall we identified and thus verified 26 of 68 putative lipoproteins (12) specifically released into the extracellular proteome of the Δlgt mutant, making *L. monocytogenes* one of the best studied species regarding lipoproteins. The only limitation of our approach seems to be the identification of lipoproteins exhibiting an additionally surface retention motif. Indeed, apart from the QoxA fragment we found none of the five lipoproteins (Lmo1379, Lmo2125, Lmo2854, Lmo2184, QoxA) featuring one or more predicted TMDs in the extracellular proteome of the Δlgt mutant. This finding might also be the result of low expression levels that rendered these genes undetectable. Encouraged by the specificity of the experimental validation of listerial lipoproteins we asked whether the deletion of *lgt* can also be exploited for systematic expression analyses of these proteins. The deletion of *lgt* should cause the release of lipoproteins into the supernatant without affecting their translocation across the membrane. In *E. coli* different prolipoproteins accumulate at the inner membrane if the lipoprotein-specific signal peptidase II (Lsp) is specifically inhibited with the cyclic peptide antibiotic Globomycin (13, 14). Prolipoproteins of *E. coli* cells treated in this way were detected at the outside of the inner membrane (15) and already carried lipid modifications (13). From these data it was concluded that (i) following translocation the prolipoproteins are retained at the membrane by their signal peptides and (ii) Lgt acts before Lsp. Indeed, inhibition of Lsp in our lgt deletion strain of *L. monocytogenes* by Globomycin revealed that signal peptide processing is required to complete the transport across the membrane. In contrast, N-terminal sequencing of released lipoproteins from untreated cultivations demonstrate that Lsp – at least in *Listeria* – can also process non-lipidated prolipoproteins. This is an important observation since it was supposed early for *E. coli* that lipidation strictly coordinates the proteolytic processing of lipoproteins (46) probably in order to
assure their retention at the bacterial membrane. An alternative (Lsp-independent) processsing by
another protease has to be considered for PrsA as suggested for B. subtilis (42) but is very
unlikely to occur systematically. In fact, no alternative processing was observed, e.g. for pre-
OppA and pre-PrtM in Lactococcus lactis lacking the Lsp activity (51). Furthermore none of the
N-terminally sequenced lipoproteins of this study exhibits the consensus sequence for proteolytic
release that was recently proposed based on data from B. subtilis (43). In conclusion, our data
indicate that inactivation of the lgt gene in L. monocytogenes results in the specific and unbiased
release of lipoproteins into the growth medium. It is very interesting to have a look on the
different functions of the verified lipoproteins. As expected half of the identified lipoproteins
belong to the group of substrate-binding proteins, emphasizing the importance of lipoprotein
metabolism in the uptake of different nutrients. We found all of the predicted di/oligopeptide-
binding proteins (OppA, Lmo0135, Lmo0153, Lmo2596) in considerable amounts in the
extracellular proteome of the Δlgt mutant. It might be assumed that these proteins are expressed
at different growth phases, depending on specific functions such as sensing environmental
changes via specific or non-specific peptides. However, it is also possible that the proteins are
expressed in parallel to guarantee an optimal nutrient supply. Proteome analyses of different
growth phases based on this novel strategy will certainly help to study the mechanism of
adaptation to environmental changes.

Our results for L. monocytogenes were used to define a genus-specific lipobox prediction
(Supplementary Table 2). The HMM did not find all annotated putative lipoproteins (68 in L.
monocytogenes EGDe) as its input is dependent on those proteins expressed under particular
growth conditions, leading to a model that will miss more divergent lipobox sequences. However,
when applied to the set of 33 Gram-positive verified lipoproteins used by Sutcliffe and
Harrington (38) all proteins were recognized, indicating that the HMM can be used for the
prediction of lipoproteins in the phylum Firmicutes. Particularly interesting is that the verified lipoprotein Lmo1068 has orthologues in both *L. innocua* and *L. monocytogenes* F2365, but the essential cysteine of the lipobox is missing in the latter proteins. Furthermore, Lmo0460 and Lmo2595 have orthologues in F2365 but not in *L. innocua*, and Lin1377, the orthologue of Lmo1340 and LMOf2365_1357, has a cysteine at position 10 and was not accepted as a lipoprotein. These four proteins are candidates for further functional studies, because all four are either only present or only lipoproteins in the pathogenic *L. monocytogenes* but not in the apathogenic *L. innocua*.

**Identification of lipoproteins regulated by PrfA**

Consequently we applied our strategy to search for additional proteins regulated by PrfA, the major regulator of *Listeria* virulence gene expression. Overall we found five proteins with clearly increased extracellular amounts in the presence of PrfA. Among these we confirmed the known PrfA-dependent expression of the virulence factors Internalin A (InlA), the actin nucleating factor ActA as well as the small, secreted Internalin C (InlC) that naturally occur in supernatant fractions of *L. monocytogenes* (49).

In addition to these expected non-lipoprotein virulence factors, we identified two lipoproteins whose expression positively correlates with PrfA: Lmo2219 is an orthologue of the post-translocation chaperone PrsA of *B. subtilis*. A positive PrfA-regulation of Lmo2219 has been demonstrated already *in vitro* (24) and at the transcriptional level *in vivo* (6, 19). The other lipoprotein was identified as Lmo0366, a protein of unknown function where the absence of a PrfA-box in the promoter region indicates an indirect PrfA regulation. In contrast to the known virulence genes, which are all absent from the genome of the closely related non-pathogenic species *L. innocua*, both, Lmo2219 and Lmo0366, have orthologues in *L. innocua*. This indicates that
Lmo0366 - as Lmo2219 - performs a surface function which is not exclusively associated with the pathogenic lifestyle of *L. monocytogenes*, but becomes more important during the infection process. The extracellular level of a third lipoprotein, Lmo2595, was significantly decreased in the presence of PrfA. Negative regulation by PrfA was reported previously, e.g., Milohanic *et al.* (24) identified eight genes which were down-regulated in parallel to an increasing PrfA expression but none of them is *L. monocytogenes* specific. Noteworthy, Lmo2595 is exclusively present in the three sequenced *L. monocytogenes* genomes (EGDe, F2365, H7858) but not in *L. innocua*, *L. ivanovii* (unpublished data), *L. welshimeri* (Hain *et al.*, J. Bacteriol., in press), and *L. seeligeri* (unpublished data). Therefore it is the first example of a protein regulated negatively by PrfA and exclusively present in the genomes of *L. monocytogenes* strains. This underscores the assumption that downregulation of Lmo2595 might be important for the infection cycle of the human pathogen. However, as gene expression of lmo2595 was inconspicuous (factor 1) in the transcriptome analysis by Milohanic *et al.* (24) a posttranslational PrfA-dependent process of regulation has to be postulated for Lmo2595.

One of the most striking differences between the differentially PrfA-expressing Δlgt strains strongly suggested a PrfA-dependent post-translational modification of another downregulated lipoprotein. We detected two forms of the oligopeptide-binding protein OppA (OppA(1) and OppA(2)), with the same apparent molecular mass but a significant difference in their isoelectric points (pI). With increasing PrfA expression OppA(2) disappears in favour of OppA(1). The PrfA-dependent modification of OppA indicates a specific role of the oligopeptide-transport system during the infection cycle of *L. monocytogenes*. Indeed, deletion of the oppA gene was shown to result in delayed growth of *L. monocytogenes* in macrophages *in vitro* as well as in organs of mice during the early phase of infection (4). A post-translational modification of OppA might be associated with a different substrate-binding capacity or regulate the binding to the
corresponding ABC-transporter complex. As the apparent pI of OppA(1) agrees with the calculated pI of 5.26 (without signal sequence), it can be assumed that OppA (2) carries the post-translational modification. Phosphorylation is very unlikely, since preliminary results obtained using the phosphospecific dye Pro-Q Diamond™ did not stain both OppA forms differentially (data not shown). Therefore, a variety of possible post-translational modifications have now to be considered and experimentally checked. The observed downregulation of oppA in the in vivo transcriptomic studies (6, 19) probably did not depend on PrfA since the total protein level of OppA (1+2) remained stable in our differentially PrfA-expressing Δlgt strains. Lmo0366 and Lmo2595 were not detected in these transcriptome studies.

In conclusion, our lgt-deletion strategy permitted a detailed study of the lipoprotein synthesis pathway in Gram-positive bacteria, demonstrated a lipidation-independent activity of Lsp and produced a comprehensive list of validated lipoproteins. The fact that we found various lipoproteins regulated by the major regulator of Listeria virulence gene expression indicates that not a single but several lipoproteins are contributing to the pathogenicity of L. monocytogenes.
ACKNOWLEDGEMENTS

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Reference List


### Table 1. Experimentally verified lipoproteins of *L. monocytogenes.*

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<th>Spot-No.</th>
<th>Protein</th>
<th>Function</th>
<th>Lipobox</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>Lmo2196</td>
<td>OppA</td>
<td>L V A C&lt;sub&gt;23&lt;/sub&gt; G</td>
</tr>
<tr>
<td>2</td>
<td>Lmo0135</td>
<td>similar to oligopeptide-binding lipoproteins, ABC transport system</td>
<td>L T A C&lt;sub&gt;20&lt;/sub&gt; G</td>
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<td>3</td>
<td>Lmo0152</td>
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<td>L T A C&lt;sub&gt;22&lt;/sub&gt; G</td>
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<td>4</td>
<td>Lmo2569</td>
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<td>L T A C&lt;sub&gt;23&lt;/sub&gt; Q</td>
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<td>5</td>
<td>Lmo1388</td>
<td>CD4+ T cell-stimulating antigen, similar to substrate-binding lipoproteins, ABC transport system</td>
<td>L G A C&lt;sub&gt;23&lt;/sub&gt; G</td>
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<td>6</td>
<td>Lmo1073</td>
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<td>7</td>
<td>Lmo2637</td>
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<tr>
<td>8</td>
<td>Lmo1068</td>
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<td>L G A C&lt;sub&gt;20&lt;/sub&gt; G</td>
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<td>9</td>
<td>Lmo2219</td>
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<td>10</td>
<td>Lmo2417</td>
<td>similar to pheromone cOB1 and substrate-binding lipoproteins, ABC transport system</td>
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<td>11</td>
<td>Lmo2331</td>
<td>similar to Gp32_bacteriophage A118 protein</td>
<td>L T G C&lt;sub&gt;22&lt;/sub&gt; G</td>
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<td>12</td>
<td>Lmo0181</td>
<td>similar to sugar-binding proteins, ABC transport system</td>
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<tr>
<td>13</td>
<td>Lmo2079</td>
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<td>I S A C&lt;sub&gt;21&lt;/sub&gt; G</td>
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<tr>
<td>14</td>
<td>Lmo0945</td>
<td>similar to C-terminal part of B. subtilis ComEC protein and to ComEA</td>
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<td>19</td>
<td>Lmo1757</td>
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<td>Lmo2431</td>
<td>similar to ferrichrome -binding lipoproteins, ABC transport system</td>
<td>L A S C&lt;sub&gt;20&lt;/sub&gt; G</td>
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<td>21</td>
<td>Lmo0541</td>
<td>similar to iron compound-binding lipoproteins, ABC transport system</td>
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<td>Lmo2416</td>
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<td>Western</td>
<td>Lmo1800</td>
<td>similar to protein-tyrosine phosphatase</td>
<td>I A G C&lt;sub&gt;22&lt;/sub&gt; G</td>
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<tr>
<td>Western</td>
<td>Lmo2595</td>
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<td>T V A C&lt;sub&gt;22&lt;/sub&gt; G</td>
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Table 2. N-terminal sequences of lipoproteins released into the supernatant of the Δlgt strain.

<table>
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<tr>
<th>Protein</th>
<th>Spot-No.</th>
<th>N-terminal sequence</th>
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<tbody>
<tr>
<td>Lmo2196 (OppA)</td>
<td>1</td>
<td>_</td>
</tr>
<tr>
<td>Lmo0135</td>
<td>2</td>
<td>_</td>
</tr>
<tr>
<td>Lmo1388</td>
<td>5</td>
<td>CGSSSDKDSS</td>
</tr>
<tr>
<td>Lmo1073</td>
<td>6</td>
<td>CGNTEVKETT</td>
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<td>Lmo2637</td>
<td>7</td>
<td>CGSSDDSSKD</td>
</tr>
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<td>Lmo2219 (acidic spot)</td>
<td>9</td>
<td>CGGGGDVVKT</td>
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<tr>
<td>Lmo2219 (basic spot)</td>
<td>9</td>
<td>ACGGGGDVVKT</td>
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<tr>
<td>Lmo2331</td>
<td>11</td>
<td>CGGTDNTRKE</td>
</tr>
<tr>
<td>Lmo1847</td>
<td>22</td>
<td>CSSQNSDSKK</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

**Figure 1:** *In vitro* infection analyses. **A.** Fluorescence microscopy of the mouse fibroblast cell line 3T3 infected (MOI of 100) with the EGDe wild-type (wt) or Δlgt strain. 3h post infection, F-actin was stained with phalloidin (green) and bacteria were labeled with anti-*Listeria* antibodies (red). **B.** Invasion and intracellular growth analyses of EGDe wild-type and Δlgt. Human epithelial cells (CACO-2) and the mouse fibroblast cell line 3T3 (3T3) were inoculated with wild-type and Δlgt at a multiplicity of infection of approximately 6. After 1h, 3h, 5h and 7h incubation in the presence of Gentamycin, cells were washed with PBS and lysed by addition of cold Triton X-100. Viable bacteria released from the cells were plated onto BHI plates to count colonies. The diagram shows the mean and standard deviation of triplicate measurements.

**Figure 2:** **A.** Expression of Lgt in EGDe wild-type (*wt*), Δlgt and the complemented strain Δlgt (pE1lgt). Total cell extracts (15µg protein) were separated by SDS-PAGE, transferred to a PVDF membrane and Lgt expression was analyzed with polyclonal anti-Lgt antibodies. **B.** Metabolic labeling of lipoproteins with [14C]-palmitic acid. *L. monocytogenes* EGDe wild-type, Δlgt and the complemented strain Δlgt (pE1lgt) were grown in the presence of [14C]-palmitic acid until the late exponential phase. Proteins were extracted by chloroform/methanol, separated by SDS-PAGE and transferred to a PVDF membrane. [14C]-palmitoylated polypeptides were analyzed by autoradiography. No labeled protein band was detectable in the extract of Δlgt.

**Figure 3:** **A:** Dual-channel image showing a comparison between the extracellular proteome of *L. monocytogenes* EGDe wild-type (orange) and Δlgt (blue). Cells of wild-type and Δlgt were
grown in BHI and harvested after reaching the stationary phase. Following precipitation with TCA/Acetone proteins were separated by 2D gel electrophoresis. Numbers indicate proteins which are detected exclusively or upregulated in the extracellular proteome of Δlgt deletion mutant. All of these proteins identified by MALDI-MS feature a typical prolipoprotein signal sequence. The numbers correspond to those in tables 1 and 2.

**B:** Dual–channel image showing comparison between the extracellular proteome of the EGDe wild-type (orange) and the complemented Δlgt (pE1lgt) strain (blue). The wild-type phenotype is completely restored in the Δlgt strain harbouring an additional plasmid with the lgt gene.

**Figure 4:** Comparison of the extracellular proteome of *L. monocytogenes* Δlgt grown without (A) and with (B) Globomycin that selectively inhibits the lipoprotein-specific signal peptidase II (Lsp). Cell cultivation and sample preparation was done as described in Figure 3.

**Figure 5:** Growth curves of *L. monocytogenes* Δlgt (pES11) (●), ΔprfA/Δlgt (pES11) (Δ) and Δlgt (pESP11prfA) (□) in minimal medium at 37°C.

**Figure 6:** Comparison of the extracellular proteome of *L. monocytogenes* Δlgt ΔprfA/Δlgt (pES11) (-prfA) and Δlgt (pESP11prfA) (+prfA). Bacteria were grown in minimal medium and Δlgt (pES11) cells were harvested at late exponential phase. Cells of the Δlgt (pESP11prfA) strain were harvested isochronal to the Δlgt (pES11) strain at an OD_{600nm} of approximately 0.4. After precipitation with TCA/Acetone, extracellular proteins were separated by 2-D gel electrophoresis as described in Material and Methods. MALDI-MS resulted in the identification of five proteins (ActA, InlA, InlC, Lmo2219, Lmo0366) with significantly increased extracellular
expression levels that correlated with an increasing PrfA expression. The oligopeptide-binding protein OppA was present in two differently regulated protein spots. The observed pI-shift suggested a PrfA-regulated post-translational modification of OppA.

**Figure 7:** A. Immunoblot analyses of the extracellular proteome of *L. monocytogenes* EGDe wild-type (wt) and Δlgt. Extracellular proteins fractions were prepared as described (legend Figure 3), separated by 2D gel electrophoresis and blotted on PVDF membranes. The blot was developed using polyclonal antibodies against the predicted lipoproteins Lmo1800 and Lmo2595. B. Immunoblot analyses of the extracellular proteome of *L. monocytogenes* Δlgt (pES11) (wt prfA), ΔprfA/Δlgt (pES11) (-prfA) and Δlgt (pEPS11prfA) (+ prfA). Extracellular proteins fractions were prepared as described (legend Figure 6), transferred to PVDF membranes using a slot blot unit and reacted with polyclonal antibodies against the predicted lipoproteins Lmo1800 and Lmo2595.
Figure 1:

A. 

B. 

- Infection 3TC
- Infection Caco-2
Figure 2:

A.

B.
Figure 3:
Figure 4:
Figure 5:
Figure 6:
Figure 7:

A. 

\[
\begin{array}{cc}
\text{Lmo1800} & \text{Lmo2595} \\
\text{wt} & \text{wt} \\
\text{\(\Delta\)lgt} & \text{\(\Delta\)lgt}
\end{array}
\]

B. 

\[
\begin{array}{cc}
\text{Lmo1800} & \text{Lmo2595} \\
+prfA & +prfA \\
wt & wt \\
prfA & prfA \\
+prfA & -prfA \\
wt & -prfA \\
prfA & -prfA
\end{array}
\]