The MprF protein is required for lysinylation of phospholipids in listerial membranes and confers resistance to cationic antimicrobial peptides (CAMPs) on *Listeria monocytogenes*.

Kathrin Thedieck¹#, Torsten Hain³#, Walid Mohamed³, Brian J. Tindall², Manfred Nimtz¹, Trinad Chakraborty³*, Jürgen Wehland¹ and Lothar Jänsch¹*

¹ Helmholtz Centre for Infection Research, Division of Cell and Immune Biology, Cellular Proteomics Group, Inhoffenstraße 7, D-38124 Braunschweig, Germany
² Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Inhoffenstraße 7b, 38124 Braunschweig, Germany.
³ Institute of Medical Microbiology, Justus-Liebig-University Giessen, 35392 Giessen, Germany.

For correspondence:
Email lothar.jaensch@helmholtz-hzi.de, Tel: (+49) 531 61813030, Fax (+49) 531 61817099; or:
Email: trinad.chakraborty@mikrobio.med.uni-giessen.de, Tel: (+49) 641 99 41250; Fax: (+49) 641 99 41259 .

# Contributed equally to this work.

**Running title:** MprF of *Listeria monocytogenes*

**Keywords:** Lmo1695, MprF, CAMP, innate immunity, survival factor
Summary

Pathogenic bacteria have to cope with defence mechanisms mediated by adaptive and innate immunity of the host cells. Antimicrobial cationic peptides (CAMPs) represent one of the most effective components of the host innate immune response. Here we establish the function of Lmo1695, a member of the VirR-dependent virulence regulon, recently identified in Listeria monocytogenes. Lmo1695 encodes a membrane protein of 98kDa with strong homology to the multiple peptide resistance factor (MprF) of Staphylococcus aureus. Like staphylococcal MprF, we found that Lmo1695 is involved in the synthesis of the membrane phospholipid lysylphosphatidylglycerol (L-PG). In addition, Lmo1695 is also essential for lysinylation of diphosphatidylglycerol (DPG), another phospholipid widely distributed in bacterial membranes. A Δlmo1695 mutant lacking the lysylated phospholipids was particularly susceptible to CAMPs of human and bacterial origin. The mutant strain infected both epithelial cells and macrophages only poorly and was attenuated for virulence when tested in a mouse model of infection. Lmo1695 is a member of a growing list of survival factors which enable growth of L. monocytogenes in different environments.
Introduction

Listeria monocytogenes is a Gram-positive, rod shaped, facultatively intracellular bacterium occurring ubiquitously in soil and water. It is taken up by humans in contaminated food and can cause serious diseases, primarily affecting pregnant women, newborns, and immunocompromised individuals (Vazquez-Boland et al., 2001). The bacterium induces its own uptake into non-phagocytic cells by specific binding to host surface receptors via the bacterial proteins Internalin A and B (Gaillard et al., 1991; Dramsi et al., 1993; Lingnau et al., 1995). In this fashion the bacterium crosses natural barriers, namely the gut epithelium as well as the blood-brain barrier and the fetoplacental barrier. Its intracellular lifestyle allows the bacterium to escape the humoral arm of adaptive immunity. However, it has still to cope with several host innate defence mechanisms such as CAMPs.

The production of pore forming cationic peptides is a common antimicrobial strategy employed by many organisms in different environments. They are widely distributed in animals and plants from which it has been inferred that they are ancient host defence factors (Hoffmann et al., 1999). Moreover, this kind of molecules is also produced by certain bacteria such as several staphylococcal strains (Bierbaum et al., 1996).

Defensins are CAMPs found in various human tissues and cell sources such as neutrophils, monocytes, macrophages, intestinal paneth cells and epithelial cells (Lehrer and Ganz, 2002; Klotman and Chang, 2006) that recognize and inactivate microbial pathogens once they attempt to infect and colonize host tissues (Medzhitov and Janeway, 2000). Based on sequence motifs and intramolecular disulfide bridges two defensin classes, α- and β-defensins, are distinguished. The α-defensins were first identified in human neutrophils (Ganz et al., 1985) where the human neutrophil peptides (HNPs) 1-4 are the most abundant proteinaceous fraction (Lehrer and Ganz, 1999; Lehrer and Ganz, 2002). They contribute to the oxygen-independent
killing of phagocytosed microbial cells. The α-defensins HD-5 (human defensin) and HD-6 are primarily found in human Paneth cells in the small intestine (Porter et al., 1997; Ganz, 1999) which produce α-defensins in response to being challenged with bacteria or inflammatory bacterial products (Ayabe et al., 2000). \textit{L. monocytogenes} has been reported to be sensitive to HNP-1 and HNP-2 (Lopez-Solanilla \textit{et al.}, 2003). In contrast to α-defensins, intestinal β-defensins have been found throughout the gastrointestinal tract (Otte \textit{et al.}, 2003). The β-defensin hBD-1 (human beta defensin) is constitutively expressed whereas hBD-2 expression must be induced by infection with enteroinvasive bacteria in human colon epithelial cell lines such as Caco-2 as well as \textit{in vivo} (O’Neil \textit{et al.}, 1999).

Bacteriocins include CAMPs of bacterial origin (Bierbaum \textit{et al.}, 1996) and are important factors in competition between closely related bacterial species in nutrient-poor environments (Riley and Gordon, 1999). Lantibiotics, one group of pore forming peptides, are produced by many Gram-positive bacteria. The lantibiotic gallidermin, produced by \textit{Staphylococcus gallinarum} shares an identical sequence with epidermin (\textit{S. epidermidis}) with the exception of a leucine residue in position 6 (Kellner et al., 1988). Members of the genus \textit{Listeria} have been shown to be sensitive to several bacterial CAMPs (Foulquie Moreno \textit{et al.}, 2003; Netz \textit{et al.}, 2001; Zheng \textit{et al.}, 1999; Gravesen \textit{et al.}, 2001).

The net negative charge of teichoic acids, peptidoglycan and phospholipids of certain Gram positive bacteria has been shown to contribute to CAMP sensitivity. Hence, CAMP resistance has been suggested to be a key virulence factor of many pathogens (Peschel and Collins, 2001). The question of how bacteria resist the action of CAMPs is currently of great interest but there are only a few reports regarding CAMP sensitivity of members of the genus \textit{Listeria}. Most lipoteichoic acid (LTA) producing bacteria achieve partial neutralization of the negatively
charged phosphate groups in the backbone by esterification of the polymers with D-alanine (Fischer, 1997).

In addition MprF - the multiple peptide resistance factor – which catalyzes the transfer of lysine residues from lysyl-tRNAs to phosphatidylglycerol (PG) (Staubitz et al., 2004; Gould and Lennarz, 1967; Lennarz et al., 1966) appears to have a major role in bacterial peptide resistance. A CAMP sensitive S. aureus mutant carrying an inactivated mprF gene lacks lysylphosphatidylglycerol (L-PG). Lysine esterifies to one of the two hydroxyl groups of PG and the free amino groups impart a net positive charge to L-PG (Peschel et al., 2001). While L-PG has been detected in S. aureus and L. monocytogenes it is also present in other members of the genus Listeria (Fischer and Leopold, 1999), several members of the genus Lactobacillus (Exterkate et al., 1971), some members of the genus Bacillus (Minnikin and Goodfellow, 1981), and members of the genus Vagococcus (Fischer and Arneth-Seifert, 1998). Human neutrophils inactivate a S. aureus mprF mutant more efficiently than the wild type suggesting an important role for L-PG in infection and in mediating CAMP resistance (Kristian et al., 2003a).

Wehmhoner et al. (2005) have recently shown that Lmo1695 is a low abundant membrane protein and it was suggested to have sequence similarity to staphylococcal MprF (Vadyvaloo et al., 2004; Dieterich et al., 2005). In this study, we have investigated the stability, localization and reaction products of the enzyme as well as its relevance for CAMP resistance and infection efficiency in vitro and in vivo. We suggest that Lmo1695 is a general survival factor in Listeria spp. imparting intrinsic resistance to cationic antimicrobials when growing in diverse environments.
Results

Lmo1695 expression is independent of PrfA

Analysis by the BLAST algorithm (Altschul et al., 1990), using the \textit{S. aureus} MprF sequence as query, revealed only one putatively orthologous sequence in the genome of \textit{L. monocytogenes} which has been designated \textit{lmo1695} (Score 306 bits, Expect value 2e-81, 26% identities, 43% positives, 5% gaps). Possible transmembrane structures of Lmo1695 were predicted using the TopPred2 algorithm (Claros and von Heijne, 1994) showing that Lmo1695’s transmembrane domains (TMD) are similar to MprF of \textit{Staphylococcus aureus} (Peschel \textit{et al.}, 2001). The highly hydrophobic N-terminal regions of Lmo1695 were predicted to contain 13 transmembrane segments, whereas the C-terminal domain is hydrophilic and predicted to be located outside the cytoplasmic membrane (see supplementary material, Fig. S1). Regulation of Lmo1695 has been predicted by DNA-based sequence analysis to be regulated by the virulence gene regulator PrfA (Glaser \textit{et al.}, 2001).

A deletion mutant for \textit{lmo1695} was constructed and used to evaluate whether Lmo1695 was involved in lysinylation of polar membrane lipids, CAMP resistance and infection efficiency of \textit{L. monocytogenes}. To detect the expression of the predicted membrane protein Lmo1695, polyclonal antibodies were raised against three different peptides corresponding to the hydrophilic portion of Lmo1695. All three antibodies were highly sensitive and selective against the respective peptides (data not shown). Bacterial membranes were purified from the wild type, the \textit{\Delta lmo1695} and the \textit{\Delta prfA} mutant by discontinuous sucrose step gradients as described previously (Wehmhoner \textit{et al.}, 2005) and separated by SDS-PAGE. Fluorescence-staining indicated that protein patterns were comparable for the wild type and mutants (Fig. 1A). The polyclonal antibodies detected Lmo1695 in membrane preparations of the wild type and \textit{\Delta prfA} mutant but not in the \textit{\Delta lmo1695} deletion mutant (Fig. 1B). A single polypeptide of 98 kDa
(calculated 97607 Da) was detected in membrane extracts indicating its association with the membrane. Lmo1965 appears to be a stable constituent of the cytoplasmic membrane as no degradation products were detected using immunoblotting with all three antibody preparations (data not shown). Membrane preparations of a ΔprfA deletion mutant showed a signal for Lmo1695 which was comparable to that of the wild type. These findings suggest that, unlike known virulence factors located at the membrane such as ActA (Fig. 1C), Lmo1695 expression is not PrfA-dependent.

A Δlmo1695 mutant poorly infects epithelial cells and macrophages, but is not affected in cell-to-cell spreading ability

The growth behaviour of the Δlmo1695 mutant and the complemented mutant strain Δlmo1695::lmo1695 was characterized in BHI medium (see supplementary material, Fig. S2). Wild type and mutants reached the same optical density although the complemented mutant reached the exponential phase slightly later than the other strains. Growth curves in minimal medium revealed that during the log phase, the wild type always grew somewhat faster than the deletion mutant although both strains attained the same final optical density (Fig. S3).

Previously, Mandin et al. (2005) reported that transcription of the lmo1695 gene is lowered in a virR mutant as compared to the parental strain and that this mutant is attenuated for infection of Caco-2 cells. We examined whether an Δlmo1695 mutant would be affected in its ability to infect two different human epithelial cell lines as well as a mouse macrophage cell line. In gentamicin survival assays using Caco-2, HeLa and the macrophage cell line P388D1, the deletion mutant was found to be significantly impaired for infection (Fig. 2). This property was restored in the complemented mutant strain. We also examined the growth properties of bacteria in the macrophage cell line P388D1, and found that despite the poor infection rates, bacteria that were
present in the cytoplasm were nevertheless able to grow intracellularly. To examine for defects in cell-to-cell spread we examined for plaque formation on L929 cell monolayers. The deletion mutant did not exhibit a significant defect for cell-to-cell spreading efficiency (data not shown).

A Δlmo1695 mutant is attenuated for virulence in the mouse infection model

To examine in vivo growth kinetics of the isogenic Δlmo1695 mutant and its complementation mutant we used a mouse model of organ colonization. As shown in figure 3, we found that Δlmo1695 mutant was present at consistently lower bacteria numbers in spleens and livers of infected mice as compared to the wild type strain at all time points examined. Complementation of lmo1695 restored survival of the deletion mutant in mice organs to wild type levels.

L-PG and L-DPG synthesis in L. monocytogenes depends on Lmo1695

Staphylococcal MprF acts as an enzyme transferring lysyl residues from lysyl-tRNA to PG (Peschel et al., 2001; Staubitz et al., 2004). In order to investigate whether the same function could be detected for Lmo1695, we analyzed and compared polar lipids from L. monocytogenes EGD-e wild type and the Δlmo1695 mutant. If Lmo1695 is indeed an MprF orthologue, lysylated PG would be found in the wild type but not in the deletion mutant.

Separation of total extracted polar lipids using two dimensional thin layer chromatography (2D-TLC) indicated the presence of a number of phospholipids and glycolipids. These were characterized by their Rf value and staining behaviour (Fig. 4). In a parallel series of experiments unstained spots were eluted from the plates and subjected to further analysis by ESI-MS/MS (electrospray ionisation – tandem mass spectrometry) and GC-MS (gas chromatography – mass spectrometry). Comparison of the 2D-TLC chromatograms of the wild type and the Δlmo1695 mutant indicated that two aminophospholipids were absent from the deletion mutant (Fig. 4). In
view of the fact that MprF is known to be responsible for the biosynthesis of L-PG in *S. aureus* and that members of the genus *Listeria* are known to contain both, L-PG and lysyldiphosphatidylglycerol (L-DPG) (Fischer and Leopold, 1999), it was necessary to confirm the identity of the two aminophospholipids (PNL 1 and PNL 2, Fig. 4A). Both were subjected to ESI-MS and MS/MS.

In Fig. 5A, the spectrum obtained from the lipid material in PNL 1 (phosphate and ninhydrin positive lipid 1) is depicted, showing major molecular ions [M+H]$^+$ at m/z 1426 (10% relative intensity), 1454 (70%) and 1482 (100%). These masses are compatible with the presence of L-DPG incorporating four C15:0, three C15:0 and one C17:0, and two C15:0 and two C17:0 fatty acid residues. The corresponding sodium adducts and smaller amounts of lipids with a single even numbered fatty acid at m/z 1440 and 1468 were also detected. These results are consistent with the distribution of fatty acids in L-DPG of *L. welshimeri* (Fischer and Leopold, 1999). To confirm the proposed structure, daughter ion spectra were obtained from the major signals. Parent ions were selected by the first mass analyser, then subjected to collision induced dissociation (CID), and the resulting fragments (daughter ions) separated by the second mass analyser. The daughter ion spectrum of the compound at m/z 1454 is depicted in Fig. 5B. From the fragmentation pattern, the structure of the molecule could be readily deduced as is shown in the inserted fragmentation scheme of Fig. 5B.

A similar analysis of the molecular ion at m/z 1482 yielded an analogous fragmentation pattern. Interestingly, the acylated glycerol moieties each contained one C15:0 and C17:0 fatty acid residue as could be deduced from the corresponding fragment. Asymmetric structures with two identical fatty acid residues bound to one glycerol were not observed (data not shown). It should be noted that ESI-MS or ESI-MS/MS does not distinguish between straight chain or anteiso-/iso-
branched chain fatty acids. Previously published work (Fischer and Leopold, 1999) indicates that the C15:0 and C17:0 fatty acids are present as anteiso- and iso-branched fatty acids. The positive ion ESI-spectrum from PNL 2 revealed major signals at m/z 823 (15%) and 851 (85%) compatible to L-PG with two C15:0, and one C15:0 and one C17:0 fatty acid residue. MS/MS analysis confirmed this assignment (data not shown).

These results strongly indicate that the two candidate spots PNL 1 and PNL 2 are lysinylated derivatives of diphosphatidylglycerol (DPG) and PG, respectively. In order to unequivocally prove that a lysine residue is linked to DPG and PG, all samples obtained from the spots on the TLC plate were subjected to GC-MS amino acid analysis after hydrolyzation and derivatization of the resulting liberated amino acids with tertiary-butyl-dimethylsilyl (TBDMS) groups to obtain volatile compounds. Fig. 5C and D show a comparison of the chromatograms from PNL 2 (L-PG, panel C) and PG (panel D). Whereas the analysis of PNL 2 yielded a peak corresponding to tris(t-butyl-dimethyl-silyl)-lysine, no trace of lysine was detected in the spot assigned to PG by ESI-MS. These data unequivocally demonstrate the presence of lysine in PNL 2 (L-PG). Lysine was also detected in material from PNL 1, confirming its presence in L-DPG. In all other spots we failed to detect significant amounts of lysine (data not shown).

Since Lmo1695 might also be involved in other lysinylation reactions, e.g. at the cell wall, peptidoglycan material from the wild type and Δlmo1695 mutant was isolated and subjected to amino acid compositional analysis. However, no significant levels of lysine in the cell wall were detected either in the wild type or the Δlmo1695 mutant making involvement of Lmo1695 in lysinylation at this site rather unlikely (data not shown).

In conclusion, Lmo1695 is responsible for lysinylation of phospholipids in L. monocytogenes. We provide direct evidence that PG is lysinylated, as reported for staphylococcal MprF (Peschel
et al., 2001). Additionally the detection of L-DPG exclusively in the wild type indicates the synthesis of this compound is directly dependent on the presence of Lmo1695.

*Lmo1695 confers resistance to gallidermin and α-defensins on L. monocytogenes*

Lysinylation is known to increase the net positive charge on the bacterial membrane. To investigate whether the detected L-PG and L-DPG levels synthesized by Lmo1695 mediate resistance to CAMPs, we tested the susceptibility of the Δlmo1695 mutant to gallidermin and α-defensins.

The effect of gallidermin on the wild type and the Δlmo1695 mutant demonstrates that the deletion mutant was already attenuated at much lower gallidermin concentrations (Fig. 6A). At 0.75 μg ml\(^{-1}\) gallidermin, growth of the deletion mutant was completely abolished whereas the wild type stopped growing entirely only at 1.25 μg ml\(^{-1}\) (Fig. 6B). Interestingly, it appeared that growth of the wild type was even induced at the subinhibitory gallidermin concentration of 0.5 μg ml\(^{-1}\) as cfu counts increased compared to plates containing lower concentrations or no gallidermin at all.

A similar effect could be reproduced in liquid culture, the deletion mutant being clearly more susceptible to gallidermin than the wild type (Fig. 7A). Whereas the deletion mutant showed growth inhibition at a concentration of 0.25 μg ml\(^{-1}\) gallidermin, the wild type was only inhibited by 1.0 μg ml\(^{-1}\). Moreover, in minimal medium liquid culture growth of neither the wild type nor the deletion mutant was completely inhibited. In both cases the maximum degree of inhibition of growth was 35 – 40% and remained constant at this value at increasing gallidermin concentrations. This suggests that saturation of the limited number of CAMP binding sites present on the bacterial surface probably plays a role.
To prove whether the effect was restricted to the bacteriocin gallidermin or if similar effects would also be observed for the human α-defensins HNP-1 and –2, we repeated the same experiment with these two CAMPs (Fig. 7B and C). At a concentration of 0.5 µg ml\(^{-1}\) growth of the deletion mutant was reduced by both, HNP-1 and 2, with the degree of inhibition being significantly greater in the deletion mutant than in the wild type.

Growth of the wild type appeared not to be inhibited by the addition of up to 1.0 µg HNP-2; on the contrary it was enhanced. At no HNP-2 concentration tested was wild type growth significantly reduced compared to growth without HNP-2. When HNP-1 was added, growth of the wild type was not affected up to 1.0 µg ml\(^{-1}\) and dropped to values similar to those of the deletion mutant at 2.0 µg ml\(^{-1}\). Surprisingly at 5 µg ml\(^{-1}\) HNP-1, cell densities which were even significantly above the levels that had been observed without HNP-1 were obtained.

Taken together these data clearly prove that the presence of Lmo1695 confers resistance to CAMPs of bacterial as well as of human origin on \textit{L. monocytogenes}.

To test whether similar effects could be seen also in rich medium we repeated the above experiment in BHI medium with increasing gallidermin concentrations (see supplementary data, figure S4). As expected the bacteria are sensitive against gallidermin also in rich medium and again the deletion mutant is less resistant compared to the wild type. Furthermore, our data demonstrate in particular the deletion mutant to be more sensitive to gallidermin in BHI than in minimal medium (>60% inhibition at 2 µg ml\(^{-1}\)) causing also a significantly higher difference in CAMP susceptibility of the wild type and the deletion mutant in BHI as compared to results obtained in minimal medium.
Discussion

Regulation of genes mediating bacterial resistance to CAMP is not entirely understood. Based on mismatch analysis Glaser et al. (2001) predicted 288 proteins which are putatively regulated by the pleiotropic virulence regulator PrfA. Lmo1695 is one of these and its predicted PrfA box was found with two mismatches. However our data suggest that Lmo1695 is not regulated by PrfA, a result that is supported by findings in a whole-genome based transcriptional study carried out by Milohanic et al. (2003) where lmo1695 was not found to be among the PrfA-regulated transcripts. It is also consistent with the recent finding that it is the VirR/VirS system which is involved in regulation of lmo1695 and the dlt operon, the latter being necessary for alanylation of LTA in L. monocytogenes (Mandin et al., 2005). Two-component systems seem to play a major role for regulation of CAMP resistance: In S. aureus D-alanine transfer to LTA appears to be own regulated by agr, a global regulator of virulence (Dunman et al., 2001). In L. monocytogenes LO28, the two-component system LisR/LisK is involved in mediating tolerance to important antimicrobials used for food preservation and in medicine (Cotter et al., 2002).

In in vitro studies with the epithelial cell lines Caco-2 and HeLa, the Δlmo1695 mutant was shown to be significantly impaired for infection. Caco-2 cells produce α-defensins 5 and 6 (Wehkamp et al., 2002; Ogle et al., 2002) and β-defensins 1 and 2 (O'Neil et al., 1999). Despite the fact that HeLa cells are not derived from the gut, they have also been found to produce defensins (Mineshiba et al., 2005) although in this cell line production of defensins have been less well characterized than in Caco-2. Infection was also impaired for the macrophage cell line P388D1 but bacteria that had accessed the host cell cytoplasm were capable of intracellular growth.

In S. aureus the dlt operon contributes to infection efficiency in human neutrophils and a mouse model (Collins et al., 2002). This has also been reported for L. monocytogenes LO28 (serotype
1/2c) in mice and for *in vitro* infection of various cell lines (Abachin *et al*., 2002). Interestingly, for *L. monocytogenes* EGD-e, a *dltA* mutant showed reduced bacterial counts in liver and spleen only 3 days post infection (p.i.) and was not significantly impaired for *in vitro* infection of Caco-2 cells. On the other hand a delta *virR* mutant showed reduced virulence in Caco-2 cells and was impaired to a greater degree in the mouse model than was the *dltA* mutant, with severely reduced bacteria counts in the liver and spleen of infected animals already at day 1 p.i. (Mandin *et al*., 2005). As both, the *dlt* operon and *lmo1695*, are regulated by VirR, it can be assumed that reduced *lmo1695* expression contributes to the reduced virulence of the *virR* mutant in Caco-2. Consistently, we observed that deletion of the *lmo1695* gene - similar to the phenotype of the *virR* deletion mutant - impairs the ability of *L. monocytogenes* to survive in liver and spleen of infected mice. A reduction of bacterial numbers in the organs was already visible at day 1 p.i. and further growth was significantly impaired at all later time points examined (Mandin *et al*., 2005). The mechanism(s) underlying the attenuation of the Δ*lmo1695* mutant during infection warrants further study. Here we provide unequivocal evidence that not only formation of L-PG but also that of L-DPG depends on the presence of Lmo1695. This finding is an important addition to the proven function of MprF in *S. aureus* where this enzyme appears only to be involved in the synthesis of L-PG. PG and DPG are widely distributed in the membrane of prokaryotes. The most abundant aminophospholipids in the members of the genus *Listeria* are the unusual phospholipids L-PG and L-DPG (Fischer and Leopold, 1999). Whereas DPG is present in *S. aureus* (Short and White, 1971), L-DPG has not been found in *S. aureus* or indeed in other members of the genus *Staphylococcus* (Nahaie *et al*., 1984; Oku *et al*., 2004; Fischer and Leopold, 1999). The enzymes involved in the synthesis of L-PG and L-DPG in members of the genus *Listeria* have not been reported to date. In *S. aureus* the transmembrane protein MprF has been shown to
catalyse the addition of lysine to PG. We investigated therefore whether Lmo1695 was involved in the same reaction. We found L-PG and L-DPG in the wild type but not in the deletion mutant Δlmo1695, whereas both contained the non-lysinyalted equivalents. The present work shows that deletion of a single gene eliminates the biosynthesis of lysinylated phospholipids in L. monocytogenes. Staphylococcal mprF expression is sufficient to confer L-PG production on E. coli (Staubitz et al., 2004; Oku et al., 2004), hence the presence of Lmo1695 alone may be sufficient to catalyze lysyl transfer in L. monocytogenes. The enzymatic mechanism for L-DPG synthesis remains to be elucidated. It is tempting to speculate that Lmo1695 might have, in addition to PG, a second substrate, namely DPG. DPG is also present in S. aureus, and such an additional function would be a result of specific changes in Lmo1695 when compared to staphylococcal MprF. Alternatively, the enzyme catalyzing the formation of DPG from PG in L. monocytogenes may also have L-PG as its second substrate. Although this appears unlikely, the third possibility is that there might even be an additional enzyme in L. monocytogenes catalyzing L-DPG synthesis from L-PG. The present data does not unambiguously allow us to determine the location of lysine in L-PG. If Lmo1695 is directly responsible for incorporating lysine into L-PG and L-DPG or that L-PG serves as the precursor of L-DPG, then a logical suggestion would be that lysine is to be found bound to the central hydroxyl group of PG, and not as previously assumed to the terminal hydroxyl group (Peschel, 2002).

We tested CAMP resistance of an Δlmo1695 mutant to both, the human α-defensins HNP-1 and HNP-2 and also to gallidermin. It is an important observation in this work that in all tests growth of the deletion mutant was inhibited to a significantly greater degree by all three CAMPs than that of the wild type. Hence, Lmo1695 confers resistance to all tested human and bacterial CAMPs on L. monocytogenes and shares this protectory effect with the staphylococcal MprF protein.
Additionally, several unexpected observations were made while monitoring the growth behaviour of *L. monocytogenes* dependent on CAMP concentration. Growth of *L. monocytogenes* is not reduced by more than 40% in minimal medium by any of the tested CAMPs although the tested gallidermin concentrations are largely within the range of the minimal inhibitory concentrations (MIC): In *S. aureus* cultivated in twofold diluted LB media MICs of 2.1 µg ml\(^{-1}\) but > 60 µg ml\(^{-1}\) were defined for gallidermin and HNP-1 respectively (Peschel *et al.*, 2001). Contrary, 2 µg ml\(^{-1}\) HNP-1 and 2 led to complete growth inhibition of *L. monocytogenes* cultivated in fourfold diluted BHI (Lopez-Solanilla *et al.*, 2003). The observed differences may be explained by the variable physiological state of the bacteria when cultivated in different media. Virulence factors and stress responses induced under starvation conditions in minimal medium may account for the higher resistance of *L. monocytogenes* towards CAMPs in the present study. Indeed, growth tests in BHI underscores the relevance of Lmo1695 for CAMP resistance by the deletion mutant that is more sensitive against gallidermin compared to minimal medium. This indicates further CAMP resistance mechanisms that are induced in particular when cultivating *Listeria* in minimal medium.

Another remarkable observation is the fact that upon addition of subinhibitory HNP-2 concentrations growth of the wild type remained not just unaffected but appeared to be even enhanced. A similar effect was observed with subinhibitory gallidermin concentrations on agar plates. This finding suggests either that subinhibitory CAMP concentrations may induce resistance mechanisms or it could be a non-specific effect, resulting for example from uptake of the peptides applied and their metabolic degradation.

We have shown that Lmo1695 is required for efficacious infection in different cell lines and in a mouse model of infection. It is directly involved in resistance to defensins and thus has a direct role in overcoming early defence mechanisms in innate immunity, as well as to the bacteriocin
gallidermin, which helps bacteria to cope with competing prokaryotes in the same ecological niche. It is in line with this finding that sequences with high similarity to MprF are not restricted to just pathogenic members of the genus *Listeria* but occur also in apathogenic strains such as *L. innocua*. Thus Lmo1695 probably has a protective role for the bacterium in a multitude of ecological habitats that these bacteria occupy.

Lmo1695 has been described recently as a part of the *vir* regulon, a second virulence regulon in addition to PrfA in *L. monocytogenes* (Mandin *et al.*, 2005). Based on the regulation of Lmo1695 expression by VirR, it appears reasonable to assume, that its function in the pathogenic strain *L. monocytogenes* is a defence mechanism against the innate immune response of the eukaryotic host. Whether it is regulated by the same or different mechanisms in response to competition from prokaryotes in the natural environment remains to be investigated.

In conclusion, we present evidence that Lmo1695 of *L. monocytogenes* EGD-e not only shows sequence similarity but also functional similarities to staphylococcal MprF. In *in vitro* infection assays, deletion of the *lmo1695* gene leads to reduction in the numbers of intracellular bacteria in several cell lines and the mutant is strongly impaired in a mouse model for listeriosis. In *L. monocytogenes* EGD-e linkage of lysine to PG and also DPG depends on Lmo1695, the latter function having not been reported for MprF of *S. aureus*. Lmo1695 confers on *L. monocytogenes* resistance to CAMPs of bacterial origin and human α-defensins. We conclude that Lmo1695 and MprF are orthologues, and based on the fact that no other sequence in *L. monocytogenes* shows the same similarity to Lmo1695 in the primary sequence of the gene and protein, we propose that it be designated MprF.
Experimental procedures

Mice

Six to eight week-old female BALB/c mice, purchased from Harlan Winkelmann (Borchen, Germany), were used in all experiments.

Strains, media and cultivation conditions

All media for bacteria cultures were sterilized by autoclaving (30 min at 121°C). For brain heart infusion (BHI) medium 37 g/L BHI (Difco Laboratories) were dissolved and ultrafiltered (cut off: 10 kDa). For agar plates 16 g/l Bacto agar (Difco Laboratories) were dissolved in medium and autoclaved. Minimal medium was always freshly prepared according to Premaratne et al. (1991).

In all experiments, fresh cultures of bacteria, prepared from an overnight culture, were used. Briefly, for infection experiments bacteria were grown in BHI, harvested in the exponential growth phase and washed twice with PBS. The pellet was resuspended in PBS and the bacterial concentration was calibrated by optical absorption. Further dilutions were prepared in PBS to obtain required numbers of bacteria for infection.

Generation of the L. monocytogenes Δlmo1695 deletion mutant and its complementation

A chromosomal in frame deletion mutant of lmo1695 was constructed by splicing by overlap extension (SOE). To create Δlmo1695 the flanking regions of the gene were amplified using PCR. The upstream region was generated by using the primers A and B (see supplementary data, Table S1) and the downstream region was synthesized with the primer pair C and D (Table S1). Purified PCR products were added into an amplification reaction with the primers A and D to generate a PCR product harboring the deletion of the gene. The PCR product was cloned into the shuttle cloning vector pCR-Blunt II-TOPO (Invitrogen) and transformed into INVαF’ E. coli
cells. After purification the plasmid harboring the cloned fragment was digested with restriction endonucleases *BamHI* and *XhoI* and ligated directly to the temperature-sensitive suicide vector pAUL-A (Chakraborty et al., 1992) digested with the restriction endonucleases *BamHI* and *SalI* and transformed into INVαF’ cells. Plasmid DNA of pAUL-A::Δlmo1695 was isolated from recombinant *E. coli* and introduced into wild type *L. monocytogenes* EGD-e as previously described by Schaferkordt and Chakraborty (1995). The in-frame chromosomal deletion was finally confirmed by sequencing the PCR product generated with oligonucleotides A and D from chromosomal DNA of the *L. monocytogenes* Δlmo1695 mutant.

Complementation of the *L. monocytogenes* Δlmo1695 deletion mutant was performed as described by Wouters et al. (Wouters, Hain, 2005). Briefly, chromosomal DNA of *L. monocytogenes* EGD-e was used to amplify a fragment spanning the region from 267 bp upstream of *lmo1696* up to 93 bp downstream of *lmo1695*. The amplified fragment was digested with *BamHI* and *XhoI* and ligated to *BamHI/XhoI*-restricted integration vector pPL2. The ligation mix was transformed into INVαF’ cells and several transformants were selected for plasmid isolation to verify the construct by DNA sequencing using oligonucleotide primers G to Q. One representative recombinant was introduced into Δlmo1695 electrocompetent cells and integration was performed and confirmed as described by Lauer et al. (Lauer, 2001).

**Preparation of polyclonal antibodies**

Polyclonal rabbit antisera were raised against three synthetic peptides derived from the sequence of Lmo1695 (amino acids 624-645, 649-668, and 677-688) and purified as described by Steffen et al. (2004). The polyclonal anti-ActA antibody was raised against the full length protein and was described elsewhere (Niebuhr et al., 1993).
Preparation of proteins from membrane extracts

50 ml of an over night BHI culture (37°C, 180 rpm) were centrifuged (6 min, 3000 g, room temperature (RT)) and pellets were resuspended in the same volume of minimal medium. After 6 h of adaptation (37°C, 180 rpm) and a washing step in minimal medium, cultures were inoculated at an OD<sub>600</sub> of 0.05. Cells were harvested (3000 x g, 8 min, 4°C) after incubation (37°C, 180 rpm) for 16 to 18 h at an OD<sub>600</sub> of 0.4 to 0.6.

The following steps were performed at 4°C; all solutions contained Protease Inhibitor (Complete, Roche). Bacterial pellets were washed with phosphate buffered saline (PBS) redissolved in 13 ml sample buffer (5 mM Mg-acetate, 100 mM K-acetate, 8 mM beta-Mercaptoethanol, 50 mM HEPES, pH 7.5 with 1 µl Benzonase (Merck, Germany), and french pressed three times (1000 psi). After centrifugation (10 min, 10000 x g) to separate nondisruptted cells sucrose was added to the supernatent to a final concentration of 0.5 M. A gradient centrifugation was performed with a 3 ml bottom layer of 2 M sucrose in sample buffer, 3 ml immediate up layer of 1.5 M sucrose in sample buffer, 8 ml sample (0.5 M sucrose) and 2 ml top layer (sample buffer without sucrose). After ultracentrifugation (100000 x g, 1 h) membranes were collected from the interphase between the immediate up layer and the sample, diluted in deionized water in a ratio of 1:6 and sedimented by ultracentrifugation at 100000 x g for 1 h. To remove cytoplasmic proteins the pellet was resuspended in 5 ml 1 M Tris ph 7.5 and incubated for 30 min on ice followed by ultracentrifugation (100000 x g, 20 min). Further washing was performed by resuspending the membranes in 100 mM sodium carbonate pH 11, sonication for 5 min on ice, incubation for 30 min on ice and ultracentrifugation (100000 x g, 20 min). This step was repeated once and followed by the same centrifugation step with 5 ml of deionized water to remove remaining sodium carbonate.
**SDS-PAGE, staining procedure and western blot analyses**

Sodium-dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with further modifications after Laemmli (1970). The fluorescent dye Ruthenium II tris (barthophamthrolin disulfonate) (RuBPS) was prepared and fluorescence staining was done according to Rabilloud et al. (2001). Western blot detection was done with LumiLight (Roche) by a CCD camera Fuji LAS-1000 (Fuji/Raytest, Germany) in a darkroom (Intelligent Dark Box, Fuji/Raytest) for up to 3 min.

**Test for resistance to cationic peptides**

On agar plates, the experiment was essentially performed as reported by Kristian et al. (2003b). 5 ml BHI precultures were washed once with minimal medium and 50 ml of minimal medium were inoculated 1/50. At OD$_{600}$ 1.0 the cultures were centrifuged (990 x g, 15 min, RT) and resuspended in 25 ml PBS. Bacteria concentration was adjusted to 1-2 x 10$^4$ cfu/ml in PBS and 30 µl were plated in triplicates for each concentration on BHI agar containing 0 - 8 µg ml$^{-1}$ gallidermin (DR PETRY genmedics GmbH, Reutlingen, Germany). After incubation for 24 h at 37°C colonies were counted and growth inhibition was determined. Experiments were done in triplicates and data were reproduced twice.

Susceptibility to defensins and gallidermin in liquid culture was determined in minimal and 0.25x BHI medium as essentially described by Lopez-Solanilla et al. (2003) and Peschel et al. (2001). The human alpha-defensins HNP-1 and HNP-2 (Sigma) were dissolved in water at a concentration of 0.1 µg ml$^{-1}$, filtered sterile and stored at −20°C. Results were reproduced twice. 50 ml cultures were inoculated as described above. Cultivation was done in triplicates in a final volume of 80 µl with peptide concentrations of 0-2 µg ml$^{-1}$ for gallidermin and 0-5 µg ml$^{-1}$ for defensins, respectively. Plates were incubated for 30 h at 37°C and bacterial growth was
monitored by measuring the OD$_{600}$ in a Bioscreen C automated reader (ThermoLabsystems, USA) at 30 min intervals. The mean value and standard deviation were calculated for the optical densities measured at the last six time points during the stationary phase. For the wild type without CAMP addition values were scaled to 100%. The purity of the HNP-1, HNP-2 and gallidermin was tested for all experiments by MALDI-MS. Only the expected molecular ions were detected (Fig. S5).

Extraction and analysis of polar membrane lipids by 2D-TLC

Bacteria were grown in minimal medium to OD$_{600}$ = 1.0 and harvested, washed, freeze dried and stored at –20°C. A modified method of Bligh and Dyer (1959) was used for the extraction of membrane lipids. 100 mg of freeze dried cell material was extracted with 9.5 ml of a chloroform:methanol (CHCl$_3$: MeOH) : 0.3% NaCl (1 : 2 : 0.8, v/v/v) at 80°C for 15 min and stirred for a further hour at RT. Cell debris was removed by centrifugation (3000 rpm, 5-10 min, RT) and the supernatant was decanted into 5 ml chloroform : 0.3% NaCl (1 : 1, v/v). After centrifugation (see above), the lower chloroform phase was collected and taken to dryness under a stream of nitrogen. Dried polar lipid material was redissolved in 250 µl of chloroform : MeOH (2 : 1, v/v) and stored at –20°C.

Silica thin-layer plates (Macherey-Nagel, Art. Nr. 818 135) were spotted with the lipid solution and run in the first dimension with chloroform : MeOH : water (65 : 25 : 4, v/v/v), and in the second dimension with chloroform : MeOH : acetic acid : water (80 : 12 : 16 : 4, v/v/v/v) as described previously (Tindall, 1990). Total polar lipids were detected by spraying with 5% dodecamolybdatophosphoric acid, ninhydrin was used for detection of amino acids, phospholipids detected by spraying with the phosphate reagent and glycolipids were identified
using α-naphtol/H$_2$SO$_4$. Further identification of the lipids was carried out by mass spectrometric analysis.

**Analysis of 2D-TLC spots by ESI- and GC-MS**

Spots were scraped from the TLC plates, extracted with 80% MeOH/20% chloroform and evaporated to dryness. The extract was redissolved in MeOH/20% water/1% HFo, and 3 µl were applied to a gold-coated nanospray glass capillary (Protana, Odense, Denmark), whose tip was placed orthogonally in front of the entrance hole of a quadrupole time-of-flight (Q-TOF 2) mass spectrometer (Micromass, Manchester, UK) equipped with a nanospray ion source. A voltage of ~1000 V was applied. For collision induced dissociation (CID) experiments, parent ions were selectively transmitted from the quadrupole mass analyser into the collision cell. Argon was used as the collision gas and the kinetic energy was set to approximately -35 eV for fragmentation of the protonated lipids. The resulting daughter ions were then separated by an orthogonal time-of-flight (TOF) mass analyser. The remaining sample was evaporated to dryness and hydrolyzed with 100 µl 4 N TFA (100 °C, 4h). After removal of the solvent, it was derivatized with 50 µl N-methyl-N-tertiary butyl-dimethylsilyl-trifluoracetamide (MBDSTFA) in 50 ml DMF containing 0.1% pyridine (1h, 80°C). Afterwards the sample was subjected to GC-MS on a ThermoFinnigan GCQ ion trap system (MS: EI mode, GC: 30 m DB5 column).

**Preparation and analysis of cell wall extracts for lysyl residues**

Cell wall extracts were prepared as described by Calvo *et al.* (2005). The cell wall pellet was resuspended in 0.75 ml PBS and the same volume of 8% SDS in PBS, incubated for 1 h at 80°C and centrifuged (20000 x g, 20 min, RT). The supernatant was discarded and the procedure was repeated. Finally, the pellet was four times washed in 2 ml of deionized water and centrifuged
(20000 x g, 20 min, RT). Pellets were stored at –20°C. An aliquot was hydrolysed (6N HCl, 100°C, 6h). After removal of the solvent the amino acids were derivatized and analysed by GC-MS as described above.

Infection experiments and bacterial enumeration

The infection efficiency of *L. monocytogenes* wild type, mutant and its complemented strain was studied in the epithelial cell lines Caco-2 and HeLa, which were cultured in MEM (Gibco, Karlsruhe, Germany). The macrophage cell line P388D1 was grown in RPMI (Gibco, Karlsruhe, Germany) for performing intracellular survival assays. Both media were supplemented with 10% fetal calf serum (FCS). Gentamicin survival assays were performed as previously described by Lingnau et al. (Lingnau et al., 1995). Incubation with bacteria was done for one hour and gentamicin containing medium was added for a 1h for Caco-2 and HeLa cells or for 1h, 3h or 5h for P388D1 cells. Experiments were carried out in triplicates.

*In vivo* growth kinetics and survival of wild type *L. monocytogenes* and its mutant derivatives were tested in a mouse infection model. Infection was performed by intravenous injection of ca. 2000 viable bacteria in a volume of 0.2 ml of PBS. At indicated time intervals, bacterial growth in spleens and livers was determined by plating 10-fold serial dilutions of the organ homogenates on BHI. The detection limit of this procedure was $10^2$ colony-forming units (CFU) per organ. Colonies were counted after 24 h of incubation at 37°C.

Statistical analysis

All data presented in this article are representative of at least three independent experiments. Significance of the represented infection data was calculated using paired Student’s *t* test and analysis of variance. Data are expressed as mean ± standard errors.
References


FIGURES

Figure 1
Figure 2

A) Caco-2

B) HeLa

C) P388D1

Legend:
- EGD-e
- Δlmo1695
- Δlmo1695::lmo1695

Y-axis:
- Relative invasion (%) or relative survival (%)

X-axis:
- Time (2h, 4h, 6h)

Significance indicated by asterisks (*).
Figure 3
Figure 4
Figure 5
Figure 6

A

B

Relative Growth (%)

0 0,5 1 1,5 2 2,5

Gallidermin (µg/mL)

EGD-e

Δlmo1695
Figure 7

A. Gallidermin

B. HNP-2

C. HNP-1

% Growth

c(Gallidermin) µg/mL

% Growth

c(HNP-2) µg/mL

% Growth

c(HNP-1) µg/mL

EGD-e

Δλmo1695
FIGURE LEGENDS

**Fig. 1.** Western blot detection of Lmo1695 and ActA in membrane extracts. Proteins of membrane extracts of *L. monocytogenes* wild type (EGD-e) and of the deletion mutants for *lmo1695* and *prfA* were separated by SDS-PAGE. Panel (A) shows a fluorescence stain of the gel. Western detection of Lmo1695 (B) shows the protein at the expected molecular mass for the wild type and the ΔprfA mutant whereas it is completely absent from the Δ*lmo1695* mutant, although a slight excess of total membrane protein was applied for this mutant as demonstrated by the fluorescence stained gel. Western detection of the virulence factor ActA (C) shows ActA to be present in the wild type and to a lesser degree in the Δ*lmo1695* mutant, whereas it is not detectable in the ΔprfA mutant proving that unlike ActA, Lmo1695 is not regulated by the PrfA.

**Fig. 2.** *In vitro* infection assays of *L. monocytogenes* wild type, Δ*lmo1695* Δ*lmo1695::lmo1695*. Effect of Δ*lmo1695* mutation and its complementation Δ*lmo1695* Δ*lmo1695::lmo1695* on intracellular bacteria numbers of *L. monocytogenes* in the epithelial cell lines Caco-2 (A) and HeLa (B) and P388D1 (C). In a gentamicin survival assay incubation with bacteria was done for 1h and gentamicin was added for a further hour for Caco-2 and HeLa and for 1h, 3h and 5h for P388D1. Bacterial numbers of Δ*lmo1695* and its complementation were compared to the numbers for the wild type that were set at 100%, providing relative percentages of infection (y-axes) for Δ*lmo1695* and Δ*lmo1695::lmo1695*. The experiment was repeated independently three times. An asterisk indicates that the means are significantly different from the wild type (*P* < 0.05).

**Fig. 3.** Kinetics of primary infection in mice with the wild type *L. monocytogenes* Δ*lmo1695* Δ*lmo1695::lmo1695*. Mice were infected i.v. with 2x10^3 of wild type *L. monocytogenes* EGD-
e, isogenic *L. monocytogenes* mutant strains Δ*lmo1695*, or the complemented strain Δ*lmo1695::lmo1695*. On days 1, 2, 3, 4, and 5 after infection, the numbers of viable bacteria in the spleens (A) and the livers (B) of three animals per group were determined. Data presented are representative of three independent experiments. An asterisk indicates that the means are significantly different from the wild type (*P* < 0.05).

**Fig. 4.** 2D-TLC analysis of total polar lipids of *L. monocytogenes* wild type (A) and Δ*lmo1695* (B). PNL 1 = phosphate and ninhydrin positive lipid 1, PNL 2 = phosphate and ninhydrin positive lipid 2, DPG = Diphosphatidylglycerol, PG = Phosphatidylglycerol, DGDG = Diglycosyl-diglyceride, GP-DGDG = Glycerophospho-diglycosyl diglyceride. Polar lipids were stained with dodecamolybdophosphoric acid. PNL 1 and 2 were not detected in the Δ*lmo1695* mutant. Mass spectrometric analysis of all spots was obtained by eluting material from an unstained plate run in parallel. PNL 1 was identified as lysyl-diphosphatidylglycerol (L-DPG) and PNL 2 as lysyl-phosphatidylglycerol (L-PG).

**Fig. 5.** ESI-MS-Analysis of PNL 1 (L-DPG). (A) Survey scan. The major signal at 1454.0 Da corresponds to the protonated mass of L-DPG with three C15 and one C17 fatty acids (double bonds not present). The mass of 1482.0 Da can be assigned to a similar molecule with two C15 and two C17 fatty acids. Additional weak signals can be assigned to low amounts of structures with one even carbon number fatty acid constituent. All L-DPG derivatives were also detected as the corresponding sodium salts. (B) Daughter ion spectrum of the molecular ion at 1454.0 Da. All detected ions are compatible with the proposed structure as shown in the inserted fragmentation scheme. (C and D) GC-MS amino acid analysis of individual 2D-TLC spots (compare Figure 4). (C) Lipid material from spots containing PNL 2 (L-PG, see figure 4) and (D) PG was subjected to GC-MS after hydrolysis and derivatization (tert. butyl-dimethyl-silylation). Depicted are the ion traces for the molecular ion of the 3-fold silylated...
lysine derivative at m/z 488. Only for PNL 2 (C) this lysine derivative was detected, whereas in the spot containing PG (D), no lysine was detectable, confirming the assignment obtained by ESI-MS. Similar data were generated for PNL 1 and L-DPG.

Branched C15 fatty acids containing no double bonds are termed br C15:0. The branched nature of the fatty acids could not be determined in the current work, but was deduced from published data in the literature.

Fig. 6. Growth assay with gallidermin. L. monocytogenes wild type (EGD-e) and Δlmo1695 were plated on BHI agar plates with increasing gallidermin concentrations. Photographs were taken after 24 h incubation at 37°C. (A) Growth at 0.5 µg ml\(^{-1}\) gallidermin. Wild type (upper picture) and deletion mutant (lower picture). (B) Quantitative representation over the whole gallidermin concentration range. Growth of the deletion mutant was inhibited at 0.5 µg ml\(^{-1}\), whereas the wild type was only inhibited at between 0.75 to 1.0 µg ml\(^{-1}\) gallidermin. Mean values of cfu counts were calculated from three replicates and expressed in %. Values for wild type were set to 100%.

Fig. 7. Growth assays in liquid culture with L. monocytogenes wild type (EGD-e) and Δlmo1695 – resistance to gallidermin (A), and the human α-defensins HNP-1 (C) and –2 (B). Minimal medium cultures with different concentrations of gallidermin, HNP-1 and –2 were inoculated with wild type and deletion mutant bacteria and cultivated for 30 h. At this time culture had reached the stationary phase. Optical density at 600 nm was measured automatically every 30 min, and the mean value and standard deviation were calculated for the optical densities measured at the last six time points during the stationary phase. Values were scaled to 100% for the wild type. Δlmo1695 mutation resulted in all cases in higher susceptibility to the cationic peptide.
Acknowledgements

This study was funded by the federal state of Lower Saxony, Germany with a Georg-Christoph-Lichtenberg fellowship within the framework of the international graduate college “Molecular Complexes of Biomedical Relevance”. This work was supported by funds obtained from the BMBF through the Competence Network PathoGenoMik (031U213B) to T.C. and T. H.

We gratefully acknowledge the excellent technical assistance of Alexandra Amend, Undine Felgenträger, Claudia Hanko, Kirsten Minkhart, Rainer Munder and Nelli Schklarenko. We thank Victor Wray and Uwe Kärst for proofreading of the manuscript.
## Supplementary material

### Table S1: Bacterial strains, plasmids and primers

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Figure S1
Figure S3

- "wild type"
- "Δlmo1695"

OD₆₀₀ vs. t (min)
Figure S4

Growth (%) after 30 h

- 0 μg/ml
- 1 μg/ml
- 2 μg/ml

[Graph showing growth in wild type and delta lmo1695 conditions at different gallidermin concentrations.]
Figure S5
Fig. S1. Alignment and comparison of transmembrane domain (TMD) distribution of Lmo1695 (L. monocytogenes) and MprF (S. aureus). TMDs are underlined and shown in red. Multiple sequence alignment was performed with CLUSTAL W (1.82) (Chenna et al., 2003). Prediction of number, orientation and position of TMDs for Lmo1695 was done using the TopPred2 algorithm, web.pasteur.fr/seqanal/interfaces/toppred (Claros and von Heijne, 1994). Data for MprF are indicated as by Peschel et al. (2001). Peptides which were used to raise polyclonal antibodies are framed. Pfam (Protein families database (Bateman et al., 2004)) domains are highlighted in grey with white fonts (DUF470), black with white fonts (DUF471) and grey with black fonts (DUF472).

Fig. S2. Growth curves for all strains used for in vitro and in vivo infection in BHI medium. The complementation mutant grew slightly slower but reached the same final optical density as the wild type and the deletion mutant.

Fig. S3. Growth curves for L. monocytogenes wild type and the deletion mutant Δlmo1695 in minimal medium. Bacteria were cultured in 50 ml minimal medium. The Δlmo1695 mutant grew slower than the wild type but reached a similar final optical density.

Fig. S4. L. monocytogenes EGD-e wild type and Δlmo1695 growth with 0, 1, and 2 µg/ml Gallidermin in BHI medium. Values are expressed in %. Growth without Gallidermin was set to 100%. At 1 µg/ml Gallidermin and below no significant growth difference was detected. At 2 µg/ml the deletion mutant appeared significantly impaired as compared to the wild type. At 4 µg/ml Gallidermin growth of both strains was completely inhibited (not shown).
Fig. S5. MALDI-MS of gallermin. Only molecular ions from gallermin were detected (2165.4 Da, 2187.4 Da and 2203.4 Da [M+H, Na, K]^+ demonstrating the purity of the applied supplement. A: Overview spectrum. B: Detailed view from the molecular ion range.