Identification of the \textit{agr} Peptide of \textit{Listeria monocytogenes}

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\textit{Listeria monocytogenes} (\textit{Lm}) is an important food-borne human pathogen that is able to thrive under a wide range of environmental conditions. Its accessory gene regulator (\textit{agr}) system was shown to impact on biofilm formation and virulence and has been proposed as one of the regulatory mechanisms involved in adaptation to these changing environments. The \textit{Lm agr} operon is homologous to the \textit{Staphylococcus aureus} system, which includes an \textit{agrD}-encoded autoinducing peptide that stimulates expression of the \textit{agr} genes via the AgrCA two-component system and is required for regulation of target genes. The aim of the present study was to identify the native autoinducing peptide (AIP) of \textit{Lm} using a luciferase reporter system in wildtype and \textit{agrD} deficient strains, rational design of synthetic peptides and mass spectrometry. Upon deletion of \textit{agrD}, luciferase reporter activity driven by the P\textsubscript{II} promoter of the \textit{agr} operon was completely abolished and this defect was restored by co-cultivation of the \textit{agrD}-negative reporter strain with a producer strain. Based on the sequence and structures of known AIPs of other organisms, a set of potential \textit{Lm} AIPs was designed and tested for P\textsubscript{II}-activation. This led to the identification of a cyclic pentapeptide that was able to induce P\textsubscript{II}-driven luciferase reporter activity and restore defective invasion of the \textit{agrD} deletion mutant into Caco-2 cells. Analysis of supernatants of a recombinant \textit{Escherichia coli} strain expressing \textit{AgrBD} identified a peptide identical in mass and charge to the cyclic pentapeptide. The \textit{Lm agr} system is specific for this pentapeptide since the AIP of \textit{Lactobacillus plantarum}, which also is a pentapeptide yet with different amino acid sequence, did not induce P\textsubscript{II} activity. In summary, the presented results provide further evidence for the hypothesis that the \textit{agrD} gene of \textit{Lm} encodes a secreted AIP responsible for autoregulation of the \textit{agr} system of \textit{Lm}. Additionally, the structure of the native \textit{Lm} AIP was identified.

Keywords: autoinducing peptide, accessory gene regulator, \textit{Listeria monocytogenes}, peptide sensing

INTRODUCTION

The Gram-positive bacterium \textit{Listeria monocytogenes} (\textit{Lm}) is an opportunist, intracellular pathogen that may cause severe, food-borne infections in high-risk groups such as immunocompromised persons, elderly people and pregnant women (Freitag et al., 2009). \textit{Lm} is able to survive and replicate in a wide range of environments including soil, various food products, and different niches inside its human host (Freitag et al., 2009; Vivant et al., 2013; Ferreira et al., 2014; Gahan and Hill, 2014). In order to adapt to these changing conditions, \textit{L. monocytogenes} possesses 15 complete two-component systems (Williams et al., 2005) and a number of regulatory circuits...
The accessory gene regulator \( (agr) \) locus encodes one of these systems and has been shown to be involved in biofilm formation, virulence and survival in the environment (Autret et al., 2003; Rieu et al., 2007; Riedel et al., 2009; Vivant et al., 2015).

The prototype \( agr \) system was described for \( S. aureus \) and consists of the four gene operon \( agrBDCA \) (Novick and Geisinger, 2008). Of the four proteins encoded by the \( agr \) operon, AgrB is a membrane-bound peptidase that cleaves and processes the \( agrD \)-derived propeptide at the C-terminus, catalyzes formation of a thiolactone ring with a central cysteine, and, in combination with the signal peptide SpsB, effects export and release of the active autoinducing peptide (AIP). Upon accumulation in the extracellular space, this AIP activates a two-component system consisting of AgrC (receptor-histidine kinase) and AgrA (response regulator). Expression of the operon is driven by the \( P_{II} \) promoter upstream of \( agrB \) and is subject to autoregulation via AgrA. Target genes of the staphylococcal \( agr \) system are either directly regulated by AgrA or by a regulatory RNAIII transcribed in the opposite direction from the \( P_{III} \) promoter adjacent to \( P_{II} \) (Thoendel et al., 2011).

Homologous \( agr \) systems have been identified in a number of Gram-positive microorganisms including streptococci, clostridia, lactobacilli, \( Bacillus \) sp., and \( Enterococcus \) \( faecalis \) (Wuster and Babu, 2008). The effects of \( agr \) regulation are pleiotropic. In \( S. aureus \), the \( agr \) system regulates a wide range of genes involved in biofilm formation, virulence, and immune evasion (Queck et al., 2008; Thoendel et al., 2011). The \( agr \) system of \( Lactobacillus plantarum \) is involved in regulation of cell morphology and adhesion to glass surfaces (Sturme et al., 2005; Fujii et al., 2008). Similar to the staphylococcal system, the \( agr \)-like \( fsr \) system of \( E. faecalis \) and the \( agr \) system of \( Lm \) are involved in regulation of biofilm formation and virulence (Autret et al., 2003; Rieu et al., 2007; Riedel et al., 2009; Cook and Federle, 2014). Moreover, in \( Lm \) more than 650 genes are directly or indirectly regulated by the \( agr \) system as shown by transcriptional profiling of an \( agrD \) deletion mutant (Riedel et al., 2009). This suggests that \( agr \) systems represent rather global regulatory mechanisms.

Despite similarities on protein level, genetic organization, and phenotypic traits regulated, known \( agr \) systems differ regarding their mechanisms of target gene regulation. While in staphylococci, a significant number of \( agr \)-dependent genes are regulated by RNAIII (Thoendel et al., 2011), no information on RNAIII transcripts are available in other organisms. In \( E. faecalis \) and \( Lm \), the genetic information upstream of the \( agr \) operon differs from that of staphylococci in that the preceding gene is transcribed in the same direction as the \( agr \) genes and no putative \( P_{III} \) promoters have been identified (Qin et al., 2001; Autret et al., 2003). Moreover, despite extensive bioinformatic approaches or transcriptional profiling a regulatory RNAIII has not been identified in \( Lm \) (Mandin et al., 2007; Toledo-Arana et al., 2009; Mellin and Cossart, 2012; Wurtzel et al., 2012). This suggests that in \( Lm \) (and \( E. faecalis \)) target genes are regulated by AgrA and/or other transcriptional regulators affected by AgrA-dependent regulation. However, it can not be excluded that the AIP signals through other two-component system besides AgrCA.

Structural information of AIPs is available only for a limited number of species. In \( S. aureus \), four \( agr \) specificity groups with different AIPs varying in size from 7 to 9 amino acids (aa) are known (Novick and Geisinger, 2008). Similarly, three \( agr \) specificity groups exist in \( S. epidermidis \) with AIPs of 8–12 aa (Otto et al., 1998; Olson et al., 2014). The AIP of \( S. intermedius \) and \( S. lugdunensis \) are 9 and 7 aa in size, respectively (Ji et al., 1997; Kalkum et al., 2003). Outside the genus \( Staphylococcus \), AIPs have been characterized for \( E. faecalis \) (11 aa), \( L. plantarum \) (5 aa), and \( C. acetobutylicum \) (6 aa) (Nakayama et al., 2001; Sturme et al., 2005; Steiner et al., 2012). Most of the known AIPs contain a thiolactone ring formed by the 5 C-terminal aa. Exceptions are the AIPs of \( C. acetobutylicum \) and \( E. faecalis \), which have ring structures consisting of 6 and 9 aa, respectively (Nakayama et al., 2001; Steiner et al., 2012). Another common feature is a central cysteine, which is replaced by a serine in some cases, required for thiolactone ring formation.

For staphylococci, \( E. faecalis \) and \( Lm \), a contribution of the \( agr \) system to virulence gene regulation has been demonstrated and \( agr \)-deficient mutants are attenuated (Riedel et al., 2009; Thoendel et al., 2011; Cook and Federle, 2014). Consequently, interference with \( agr \) signaling was proposed as a therapeutic approach (Gray et al., 2013). Of note, the specificity of the interaction between the AIP and its cognate receptor AgrA has been used to device improved strategies by fusing the AIP to a bacteriocin to induce lysis of the targeted bacteria (Qiu et al., 2003). The structure of the native AIP of \( Lm \) has not been elucidated so far. With the present study, we aim closing this gap in order to further elucidate the components and mechanisms of the \( agr \) autoregulatory circuit of \( Lm \) and to facilitate future studies on strategies to interfere with cell–cell communication of this important human pathogen.

**MATERIALS AND METHODS**

### Bacterial Strains and Culture Conditions

All strains and plasmids used in this study are listed in Table 1. \( L. monocytogenes \) was generally incubated in Brain Heart Infusion broth (BHI, Oxoid Ltd) at 30°C. \( E. coli \) strains were grown in lysogeny broth (LB). For solid media, 15 g/l agar were added to the broth before autoclaving. Antibiotics were added if necessary. Where appropriate, kanamycin was used at a final concentration of 50 (for \( E. coli \) strains) and 15 \( \mu \)g/ml chloramphenicol was used for both species. For \( Lm \) strains carrying a chromosomal copy of pPL2 derivatives chloramphenicol was used at 7 \( \mu \)g/ml.

### Generation of Recombinant Strains

Primers used for cloning or sequencing purposes are listed in Table 2. To study transcriptional activity of the \( agr \) operon, the \( P_{II} \) promoter upstream of \( agrB \) (Rieu et al., 2007) was amplified with Phusion® polymerase (Thermo Fisher Scientific) using primers PII_fwd_SalI and PII_rev and chromosomal DNA of \( Lm \) EGD-e wildtype (WT) as template. The obtained PCR fragment was digested with \( SalI \) and cloned in frame in front of the luciferase reporter into \( SalI/SwaI \)-cut pPL2lux.
Plasmids into electrocompetent provider (Eurofins, Germany). The plasmid was transformed sequencing of the PCR fragment by a commercial service primers PII_fwd_SalI and luxA_rev with subsequent Sanger analysis and amplification of the cloned PII. In both strains, successful chromosomal integration of pPL2uxP1 at the correct site (tRNAArg) was verified using primers PL95 and PL102 (Lauer et al., 2002).

For homologous overexpression of agrBD, a PCR fragment containing both genes was amplified using primers NZagrBD_fwd and NZagrBD_rev and chromosomal DNA of Lm EGD-e as template. The PCR product was digested with NcoI and SacII and ligated as exact transcriptional fusion to the constitutive P44 promoter into NcoI/SacII digested pNZ44 (McGrath et al., 2001) to yield pNZ44agrBD. The product was transformed into E. coli DH10B. Clones were screened for plasmid containing the correct insert by PCR using primers NZ-confirm_fwd and NZ_colony_rev and sequencing of the PCR product. The correct plasmid as well as the empty vector (pNZ44) were transformed in electrocompetent Lm ΔagrD generated as described previously (Monk et al., 2008).

For heterologous AIP production, agrBD or agrB alone were amplified using primer pairs agrBD_NdeI_fwd/ agrBD_BamHI_rev and chromosomal DNA of Lm EGD-e WT or ΔagrD. Following restriction with NdeI and BamHI both PCR products were ligated into NdeI/BamHI digested pET29a(+) (Merck Millipore). This fuses the PCR products to the T7 promoter creating pET29a_agrB and pET29a_agrBD,

### TABLE 1 | Bacterial strains and plasmids used in the present study.

| Strain/plasmid | Characteristics | Reference/source |
|----------------|----------------|-----------------|
| Escherichia coli DH10B | Cloning host | Thermo Fisher Scientific |
| E. coli BL21 DE3 | Used for protein overexpression | New England Biolabs |
| E. coli BL21 DE3 pET29a_agrB | IPTG-inducible expression of agrB, Kan’ | This study |
| E. coli BL21 DE3 pET29a_agrBD | IPTG-inducible expression of agrBD, Kan’ | This study |
| Listeria monocytogenes EGD-e ΔagrD | In-frame deletion of agrD in strain EGD-e | Becavin et al., 2014 |
| L. monocytogenes ΔagrD::pMM2agrD | pMK2agrD integrated into the tRNAArg locus in the EGD-e chromosome, Kan’ | Riedel et al., 2009 |
| L. monocytogenes EGD-eΔagrD:pPL2uxP1 | pPL2uxABCDEP1 integrated into the tRNAArg locus in the EGD-e chromosome, Cm’ | Riedel et al., 2009 |
| L. monocytogenes EGD-e ΔagrD::pPL2uxP1 | pPL2uxP1 integrated into the tRNAArg locus in the EGD-eΔagrD chromosome, Cm’ | This study |
| L. monocytogenes EGD-e ΔagrD pNZ44agrBD | Strain with constitutive, P44-driven expression of agrB and agrD, Cm’ | This study |
| pPL2ux | Site-specific integrative vector to study promoter activity in L. monocytogenes, Cm’ | Bron et al., 2006 |
| pPL2uxP1 | Site-specific integrative vector for P1 promoter activity analysis, Cm’ | This study |
| pNZ44 | Plasmid for constitutive gene expression driven from the lactococcal promoter P44 | McGrath et al., 2001 |
| pNZ44agrBD | Plasmid for constitutive P44-driven expression of agrB and agrD in L. monocytogenes, Cm’ | This study |
| pET29a(+) | Plasmid for strong IPTG inducible expression in E. coli, Kan’ | Merck Millipore |
| pET29a_agrB | IPTG-inducible expression of agrB in E. coli, Kan’ | This study |
| pET29a_agrBD | IPTG-inducible expression of agrBD in E. coli, Kan’ | This study |

### TABLE 2 | Primers used in this study.

| Name | Sequence | Reference/source |
|------|----------|-----------------|
| PII_fwd_Sall | CTGATGTCGACCTTTGAACAGAACAAGAAGC | This study |
| PII_rev | CAACATAATCCACCTCAGAATTATTTACACG | This study |
| luxA_rev | TACCTCTGTGGAGAAATTTGGAAGG | This study |
| PL95 | ACATAATCCACCTCAGAATTATTTACACG | Lauer, 2002 |
| PL102 | TATGAGCTCAGCCACATCC | Lauer, 2002 |
| NZagrBD_fwd | AATTGCAATGGGATTTTATCTGGAAAAAGTCCC | This study |
| NZagrBD_rev | GCATGAGATCTTATTTATCTGGAAAAAGTCCC | This study |
| NZ-conf_fwd | CCATACAGGAGAAGGACATAGAAG | This study |
| NZ-colony_rev | CTCGTGGACGGATGGGAGAAG | This study |
| agrBD_NdeI_fwd | GGAATTCCTATGAGTAGTTATATATGAGTCTGAAAAAGTCCC | This study |
| agrBD_BamHI_rev | CGGGGATCCATATCCGCTCGGTCTCCTAAAAATCTCATTAT | This study |
respectively. Both plasmids were verified for correct cloning by restriction analysis and Sanger sequencing of inserts.

**Luciferase Reporter Assays**

For luciferase reporter assays, growth experiments were performed in white 96-well microtiter plates with transparent bottom (BRANDplates® pureGrade™). A single colony was inoculated into BHI and grown over night (o/N; i.e., approx. 16 h). Following o/N growth, cultures were diluted to an optical density at 600 nm (OD$_{600}$) of 0.01 in fresh, sterile BHI. For co-cultivation of AIP producer and reporter strains, o/N cultures of both strains were used to inoculate BHI medium to a OD$_{600}$ of 0.01 and then mixed at a 1:1 ratio. 200 µl aliquots of this mix were transferred into individual wells of the microtiter plates (each condition in triplicates). Plates were incubated at 30°C in a Tecan Infinite M200 plate reader and OD$_{600}$ and luminescence intensity were measured every hour.

**Synthetic Peptides**

Synthetic peptides were purchased from Peptide Protein Research Ltd (UK) in lyophilized form with >70% purity. Peptides were reconstituted in dimethyl sulfoxide (DMSO) at 2 mM and stored at −20°C until further use. For experiments, these stocks were diluted as appropriate in 25% (v/v) DMSO in phosphate-buffered saline (PBS) to give the final concentrations as indicated. To test the effect of peptides on P$_{11}$ activity, reporter strains (Lm EGD-e::pPL2luxP$_{11}$ or ΔagrD::pPL2luxP$_{11}$) were grown o/N and diluted to an OD$_{600}$ of 0.01 in fresh BHI. 180 µl aliquots were distributed in 96 well microtiter plates (each condition in triplicate) and incubated at 30°C for 2 h. At this stage, 20 µl of diluted peptides were added to obtain the indicated final concentrations (5 nM–50 µM) and plates were incubated at 30°C in a Tecan Infinite M200 plate reader with hourly OD$_{600}$ and luminescence intensity measurements.

**AIP Production in E. coli**

For heterologous AIP production, pET29a_agrB or pET29a_agrBD were transformed into E. coli BL21(DE3) (New England Biolabs) and transformants were selected on LB agar containing kanamycin. Four single colonies were streaked onto two LB agar plates containing kanamycin with or without 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). A clone showing good growth in the absence of IPTG but reduced growth in its presence was selected and a single colony was inoculated into 5 ml of LB medium and grown o/N on a rotary shaker at 37°C. Using the o/N culture, 500 ml LeMaster and Richards minimal medium (Paliy and Gunasekera, 2007) containing 50 mM glucose were inoculated to a final OD$_{600}$ of 0.1 and incubated on a rotary shaker at 37°C to an OD$_{600}$ of 0.8. At this stage, expression was induced by addition of 1 mM IPTG. Following incubation under the same conditions for an additional 2 h, bacterial cells were pelleted via centrifugation (3000 × g, 30 min and 4°C) and supernatants were collected, filter sterilized, frozen in liquid nitrogen and lyophilized. Lyophilized samples were stored at −20°C until further analysis by LC–MS/MS.

**LC–MS/MS Analysis**

The lyophilized supernatants of recombinant E. coli strains were reconstituted in a 25:35:35:5 H$_2$O:Isopropanol:CH$_3$CN:HCOOH mixture and diluted 1:10 in H$_2$O. 5 µl were injected into a reverse-phase column with corresponding guard column (Aeris™ PEPTIDE 3.6u XB-C18 150 × 2.1 mm, Security-Guard™ ULTRA 2 × 2.1 mm guard column, Phenomenex). A constant flow rate of 0.4 ml/min was applied. Mobile phase A consisted of water with 0.2% (v/v) formic acid and mobile phase B was acetonitrile with 0.2% (v/v) formic acid. Elution program was: isocratic hold at 5% B for 5 min followed by a linear gradient from 5 to 45% B over 80 min. After each sample, the column was washed with 90% B for 10 min and equilibrated at starting conditions. Data was obtained in positive auto MS/MS mode on an Agilent 6540 Accurate-Mass Quadrupole (LC-Q-TOF/MS)
with ESI Jet Stream Technology using the following conditions: drying gas flow rate of 10 l/min with a gas temperature of 250°C, nebulizer with 40 lb per square inch gauge, sheath gas flow rate of 10 l/min, sheath gas temperature of 300°C, capillary voltage of 4000 V, and fragmentor voltage of 170 V. The collision energy was set by formula with 4.5 slope and 10 offset. Data analysis was performed using Mass Hunter Workstation Software (Ver.B.05.519.0, Agilent Technologies) and the “Find compounds by formula” algorithms. Synthetic peptides were analyzed using the same conditions as the recombinant peptides expressed in E. coli to compare retention time, accurate mass and fragmentation patterns.

**Invasion Assay**

Invasion of Lm into Caco-2 cells was tested using a standard gentamycin protection assay essentially as described previously (Riedel et al., 2009). Briefly, Caco-2 cells were cultured in DMEM supplemented with 10% (v/v) fetal calf serum (FCS), 10 mM L-glutamine, 1% (v/v) penicillin/streptomycin and 1% (v/v) non-essential amino acids (NEAA) at 37°C and a 5% CO₂ atmosphere. Cells were seeded to a density of 2 × 10⁵ cells per well in a 24 well plate and cultivated to a monolayer for 4 days. One day prior to the experiment, culture media without antibiotics was added. A fresh o/N culture of the indicated bacterial strains was diluted 1:10 in 10 ml fresh BHI and grown to mid-exponential phase (OD₆₀₀ = 0.8). Where appropriate, peptide R5T0 was added (5 µM final concentration). Bacteria were pelleted and diluted in DMEM containing 10 mM L-glutamine and 1% NEAA to 10⁶ colony forming units per ml (cfu/ml) (OD₆₀₀ = 0.5). 1 ml of this suspension was added to Caco-2 cells in quadruplicates (MOI = 100). Cells were incubated for 1 h to allow invasion of bacteria. To kill remaining extracellular bacteria, cells were washed once with PBS and 1 ml DMEM containing 10 µg/ml gentamicin (Gibco®) was added to the cells. After 1 h of incubation, cells were washed twice with PBS, lysed with ice-cold water and cfu/ml were determined by plating serial dilutions on BHI agar.

**Statistical Analysis**

All experiments were conducted in at least three biological replicates. Results were analyzed by Student's t-test or ANOVA with Bonferroni post-test analysis to correct for multiple comparisons using GraphPad Prism (version 6) as indicated in figure legends and Supplementary Data Sheet 1. Differences between different strains or conditions were considered statistically significant at p < 0.05.

**RESULTS**

**P_{II}-Activity in Lm EGD-e**

P_{II} promoter activity was analyzed in Lm EGD-e::pPL2luxP_{II} and ΔagrD::pPL2luxP_{II} during growth in BHI medium at 30°C (Figure 1A). No differences in growth or final OD₆₀₀ were observed between the two strains ruling out an effect of growth on luciferase activity. In the WT background, a significant increase in P_{II}-dependent luciferase activity was observed during exponential growth with a peak in late exponential phase. By contrast, no luminescence above background could be detected.
for the agrD-deficient strain throughout the experiment. This suggests that the AIP is required for transcriptional activity of P_{II}. AIPs are usually secreted into the extracellular environment. In order to confirm that the AIP of Lm is acting as an extracellular peptide, similar growth experiments were conducted using co-incubation of AIP producer and reporter strains in different combinations (Figure 1B). As expected, the agrD-deficient reporter strain showed no P_{II} activity when incubated with Lm EGD-e ΔagrD. However, high levels of luminescence were observed using the same reporter strain in combination with Lm ΔagrD pNZ44aagrBD, a ΔagrD derivative expressing agrBD from the P44 promoter on pNZ44. Luminescence in this setup was significantly higher compared to co-cultures of the WT reporter with the agrD deletion mutant or the agrD-deficient reporter strain with Lm EGD-e pNZ44 (i.e., the empty vector control) suggesting that AIP levels produced by Lm ΔagrD pNZ44aagrBD are higher than that of the WT.

**P_{II} Activation by Synthetic AIP Candidates**

Upon several attempts we were unable to identify the active AIP in supernatants of Lm EGD-e WT or the AIP overproducing strain ΔagrD pNZ44aagrBD grown in either BHI or modified Welshimer’s broth. Sequence alignment of AIPs with a resolved structure, revealed that most AIPs consist of a 5 aa thiolactone ring with N-terminal tail varying from 0 to 7 aa (Figure 2A). Using this information, a range of peptides based on the AgrD sequence of Lm EGD-e were synthesized consisting of a thiolactone ring of 4–6 aa and an N-terminal tail of 0–5 aa (Figure 2B). The effect of these peptides on P_{II}-driven luciferase activity was tested using the reporter strains Lm EGD-e:pPL2luxP_{II} and ΔagrD:pPL2luxP_{II}. At 5 µM, none of the peptides had a measurable effect on growth of the reporter strains (Supplementary Figures S1A,B). The peptide R5T0 consisting of a 5 aa thiolactone ring with no N-terminal tail slightly increased P_{II}-driven luminescence in the WT reporter strain during the first 4 h of the experiment (Figure 3A). However,
As observed previously, deletion of *agrD* in *L. plantarum* effects more than 600 genes and phenotypically affects biofilm formation and virulence in *in vitro* and *in vivo* (Rieu et al., 2007; Riedel et al., 2009). The best studied AIP system is the *agr* system of *S. aureus* (Wuster and Babu, 2008). In order to check if *R5T0* is not only able to induce PII activity but also functionally complement the *agrD* mutant, invasion assays were performed with *Lm* EGD-e *ΔagrD* in the presence and absence of R5T0 (Figure 4). As observed previously, deletion of *agrD* results in reduced invasion into Caco-2 intestinal epithelial cells and this defect was genetically complemented by integration of pIMK2*agrD*, i.e., a plasmid for constitutive expression of *agrD* (Riedel et al., 2009). More importantly, growth in the presence of 5 μM R5T0 completely restored invasion of *Lm* EGD-e *ΔagrD* to WT levels.

**Heterologous Production of the Lm AIP in E. coli**

In a further approach to identify the AIP of *Lm*, the *agr* genes were expressed in *E. coli* using the IPTG-inducible pET29a system. Using LC–MS, a prominent signal was identified in supernatants of an induced culture of *E. coli* BL21 pET29a*agrBD* (Figure 5A) with a mass of 627.2549 (Figure 5B). This signal was absent in the non-induced culture or supernatant of a control strain only expressing *agrB* (Supplementary Figure S2). In order to confirm the identity of the overexpressed peptide, analysis of the PII-activating synthetic peptide R5T0 was performed. Interestingly, the chromatogram of R5T0 yielded two peaks in close vicinity (Figure 5A). Both peaks correspond to peptides with identical mass and fragmentation pattern (Figure 5B). However, the different retention times and peak areas indicate that the two peaks represent stereoisomers or conformational isomers at different concentrations.

The peptide present in the supernatant of *E. coli* BL21 pET29a*agrBD* and both peaks of R5T0 had almost identical global masses (Figure 5B). Moreover, all three peptides showed highly similar fragmentation patterns (Figure 5B) and several signals of the MS/MS spectra correspond to fragments of R5T0 at a mass accuracy better than 2 ppm (Table 3; for corresponding structures see Figures 5C,D). These results clearly indicate that the listerial AIP is a cyclic pentapeptide with the amino acid sequence CFMFV forming a thiolactone ring, i.e., the structure of the synthetic peptide R5T0.

**Specificity of the Lm AIP**

Known AIPs differ greatly in sequence, length and structure among species and even strains (Figure 2A) and different AIPs of *S. aureus* display cross-inhibition (Ji et al., 1997). Similar to the AIP of *Lm*, the AIP of *L. plantarum* is a cyclic pentapeptide yet with a different sequence (Sturme et al., 2005). Further experiments were performed to test if PII activation is specific for the *Lm* AIP or if the *L. plantarum* AIP is also able to activate PII (Figure 6). As observed in the previous experiments, R5T0 slightly enhanced PII-driven luciferase activity in *Lm* EGD-e::pPl2luxPII (Figure 6A) and was a potent inducer of PII activity in the AIP-negative reporter strain *Lm* EGD-e *ΔagrD::pPl2luxPII*. By contrast, in both reporter strains the *L. plantarum* AIP had no effect on PII activity.

**DISCUSSION**

Signaling peptides, also referred to as AIPs, are produced by a wide range of Gram-positive microorganisms (Wuster and Babu, 2008) and serve various purposes (Thoendel and Horswill, 2010). The best studied AIP system is the *agr* locus of *S. aureus* and homologous systems have been identified in a variety of Gram-positives (Wuster and Babu, 2008). In *S. aureus*, the *agr* system is a rather global regulatory circuit affecting a large number of genes and different phenotypic traits (Thoendel et al., 2011). Similarly, deletion of *agrD* in *Lm* affects more than 600 genes and phenotypically affects biofilm formation and virulence in *in vitro* and *in vivo*.
et al., 2009). However, while absence of agr signaling is linked with enhanced biofilm formation of S. aureus (Vuong et al., 2000), agr mutants of Lm display reduced biofilm formation under the conditions monitored (Rieu et al., 2007; Riedel et al., 2009).

Previous studies have already indicated that, like the staphylococcal system, the Lm agr locus is subject to positive autoregulation involving a diffusible factor, probably the agrD-encoded AIP involved in regulation. Transcription levels of the agr operon were greatly reduced in agr-deficient Lm mutants (Rieu et al., 2007; Riedel et al., 2009; Garmyn et al., 2012). Also, the biofilm defect of a ΔagrD mutant was complemented when bacteria were grown in the reconstituted culture supernatants of the WT or in the presence of small amounts of WT bacteria (Riedel et al., 2009). The presented results further strengthen the hypothesis that agrD encodes a secreted AIP that positively regulates the agr system of Lm. In the ΔagrD mutant, no activity of the agr promoter could be observed (Figure 1A) and promoter activity was restored when the agrD-deficient reporter strain was co-cultured with a strain carrying a plasmid for constitutive expression of agrBD (Figure 1B).

The presented results provide further evidence that, in Lm, agrD actually encodes the propeptide, which is processed released...
into the extracellular environment where it acts as an AIP. Moreover, our data suggests that the native AIP is a cyclic pentapeptide RST0 consisting of the amino acids (from N- to C-terminus) Cys, Phe, Met, Phe, Val. A peptide with this structure was found in the culture supernatant of a recombinant E. coli strain expressing AgrBD (Figure 5) and a synthetic peptide with identical structure was able to potently induce activity of the PII promoter of the agr system (Figure 3) and to functionally complement the invasion defect in a ΔagrD mutant (Figure 4).

Induction of luciferase activity in the ΔagrD reporter upon co-cultivation with the AIP producing WT strain (Figure 1B) indicates that at least some of the AIP must be present in culture supernatants. However, we were unable to identify the native peptide in supernatants of Lm EGD-e grown in complex media (brain heart infusion) or modified Welshimer’s broth. This may be explained by the high levels of peptides in brain heart infusion, which makes identification impossible by LC–MS/MS. In modified Welshimer’s broth Lm only grows to low final optical densities and thus any secreted peptide will also be present at low concentrations especially when subject to positive autoregulation and fully induced only at high cell densities. Further studies will be needed to quantify actual AIP concentrations in culture supernatants and the threshold required to activate PII and target gene regulation.

Interestingly, four different synthetic peptides with a five-membered thiolactone ring and varying tail length had inhibitory activity on the agr promoter in the WT reporter strain, which itself is able to produce the native AIP. Since agr mutants of Lm display attenuated virulence (Autret et al., 2003; Riedel et al., 2009), this suggests that these peptides are antagonists of the native AIP and may represent a potential supplementary or alternative therapeutic approach as proposed for S. aureus and other pathogens (Gray et al., 2013). Interestingly, they also exhibited PII activation in the ΔagrD reporter to varying degrees. This may indicate that these peptides compete with RST0 or the native AIP for binding to the receptor but their affinity and/or activity is lower. Thus, of the four candidate peptides, the best antagonist of the native AIP is probably RST2, which efficiently blocks PII activity in the WT but activates luminescence only marginally in the mutant reporter.

A striking difference between the agr systems of S. aureus and Lm is the structural diversity of the AIPs. Within the species S. aureus, four specificity groups of strains with different AIP are found and these groups show cross-inhibition (Novick and Geisinger, 2008). By contrast, the AgrD propeptides of the genus Listeria are rather conserved and the species Lm, L. innocua, L. ivanovii, L. welshimeri, L. seeligeri, and L. marthii have identical (predicted) AIP sequences (Supplementary Figure S3A) suggesting cross-reactivity. Moreover, phylogenetic analysis

### Table 3 | Mass-charge-ratios (m/z) of peptide fragments detected by MS/MS and difference to the m/z calculated according to the formula of the corresponding RST0 fragment.

| Fragment | Formula | m/z (calculated) | m/z (measured)a | Difference (ppm) | Difference (mDa) |
|----------|---------|------------------|-----------------|-----------------|-----------------|
| b215     | C29H23N2O4S2 | 529.19377 | 529.1933 | −0.9 | −0.47 |
| b215-CO  | C29H23N2O4S2 | 501.19886 | 501.1994 | 1.1 | 0.54 |
| b256     | C17H22N2O2S2 | 382.12536 | 382.1256 | 0.6 | 0.24 |
| b214     | C17H22N2O2S2 | 251.08487 | 251.0843 | −2.3 | −0.57 |
| b213-CO  | C17H22N2O2S2 | 223.08996 | 223.0898 | −0.7 | −0.16 |

*aMeasured m/z are derived from the MS/MS analysis shown in Figure 5B.
Based on 16S rRNA gene sequences reveals that *Listeria* sp. that share identical AIP sequences form a cluster that separates from the other species indicating that they are more closely related (Supplementary Figure S3B).

With the exception of *C. acetobutylicum*, phylogenetic trees calculated using concatenate AgrA, AgrB, AgrC, and AgrD sequences are in line with trees inferred from 16S sequences (Wuster and Babu, 2008). This suggests that *agr* systems are generally inherited vertically. It has been proposed that *C. acetobutylicum*, whose AgrD sequence is almost identical to that of *Listeriaceae*, is the only known case of horizontal transfer of an *agr* system (Wuster and Babu, 2008). Further experimental data comparing the *Lm* AIP with the AIP of *L. plantarum*, which also consist of a five cyclic pentapeptide although with different aa composition, indicates that the *Lm* agr system is specific for the AIP of those *Listeria* sp. that share a conserved AgrD sequence but does not respond to the cyclic pentapeptide AIPs of other organisms. This also suggests that intervention strategies based on antagonistic peptides targeting the *agr* systems of *Lm* (and other organisms) are specific for organisms with identical AIPs.

In summary, the present data shows that the AgrD of *Lm* EGD-e encodes a secreted peptide consisting of a five-membered thiolactone ring, which has autoinducing activity. Moreover, the identification of several synthetic peptides with antagonistic activity proposes a potential option to treat *Lm* infections or inhibit biofilm formation as suggested by others previously.

**AUTHOR CONTRIBUTIONS**

CR conceived the study. MZ, MW, and AS-K carried out experiments. MZ, AS-K, BB, and CR analyzed data. MZ, AS-K, BB, and CR drafted the manuscript and all the authors contributed to preparing the final version of the manuscript. All authors read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00989

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