A detailed view of the intracellular transcriptome of *Listeria monocytogenes* in murine macrophages using RNA-seq

Tilman Schultze¹, Rolf Hilker¹², Gopala K. Mannala¹, Katrin Gentil¹, Markus Weigel¹, Neda Farmani¹, Anita C. Windhorst³, Alexander Goesmann², Trinad Chakraborty¹ and Torsten Hain¹*

¹ Institute of Medical Microbiology, Justus Liebig University, Giessen, Germany, ² Bioinformatics and Systems Biology, Justus Liebig University, Giessen, Germany, ³ Institute of Medical Informatics, Justus Liebig University, Giessen, Germany

*Correspondence:
Torsten Hain
torsten.hain@mikrobio.med.uni-giessen.de

INTRODUCTION

*Listeria monocytogenes* is a ubiquitous bacteria and causative agent for the foodborne infection listeriosis, which is mainly a threat for pregnant, elderly, or immunocompromised individuals. Due to its ability to invade and colonize diverse eukaryotic cell types including cells from invertebrates, *L. monocytogenes* has become a well-established model organism for intracellular growth. Almost 10 years ago, we and others presented the first whole-genome microarray-based intracellular transcriptome of *L. monocytogenes*. With the advent of newer technologies addressing transcriptomes in greater detail, we revisit this work, and analyze the intracellular transcriptome of *L. monocytogenes* during growth in murine macrophages using a deep sequencing based approach. We detected 656 differentially expressed genes of which 367 were upregulated during intracellular growth in macrophages compared to extracellular growth in Brain Heart Infusion broth. This study confirmed ~64% of all regulated genes previously identified by microarray analysis. Many of the regulated genes that were detected in the current study involve transporters for various metals, ions as well as complex sugars such as mannose. We also report changes in antisense transcription, especially upregulations during intracellular bacterial survival. A notable finding was the detection of regulatory changes for a subset of temperate A118-like prophage genes, thereby shedding light on the transcriptional profile of this bacteriophage during intracellular growth. In total, our study provides an updated genome-wide view of the transcriptional landscape of *L. monocytogenes* during intracellular growth and represents a rich resource for future detailed analysis.

**Keywords:** *Listeria monocytogenes*, intracellular, mRNA transcriptome analysis, RNA-seq, human pathogenic bacteria
from severe complications (Hamon et al., 2006; Allerberger and Wagner, 2010; Cossart and Lebreton, 2014; Elinav et al., 2014). Upon ingestion of contaminated food, *L. monocytogenes* reaches the gastrointestinal tract where it invades epithelial cells. Via infection of macrophages and escape from their phagosome, *L. monocytogenes* ultimately reaches liver and spleen. Due to its versatile ability of infecting mammalian and invertebrate cells, *L. monocytogenes* has become a popular model organism for intracellular pathogenicity (Hamon et al., 2006; Lecuit, 2007; Cossart and Toledo-Arana, 2008; Cossart, 2011).

Recently, non-coding RNA elements were frequently studied in *L. monocytogenes* (Mellin and Cossart, 2012). Non-coding RNA elements are widely implicated in adaptation to different environmental conditions, metabolism, and virulence. The role of regulatory RNA elements (Johansson et al., 2002; Mansjö and Johansson, 2011), small RNA (Christiansen et al., 2006; Mandin et al., 2007; Nielsen et al., 2008; Oliver et al., 2009; Toledo-Arana et al., 2009; Mraheil et al., 2011; Nielsen et al., 2011; Sievers et al., 2014, 2015) and antisense RNA (Toledo-Arana et al., 2009; Wurtzel et al., 2012; Behrens et al., 2014; Wehner et al., 2014) was investigated. A new class of regulatory elements, the excludon was defined as long transcript that function as both sense transcript for one gene (or set of genes) as well as antisense transcript for a divergently oriented neighboring gene (or set of genes; Wurtzel et al., 2012).

Intracellular growth of *L. monocytogenes* was included as experimental condition in several genome-wide transcriptional profiling studies, however, few publications have focused on the changes in the mRNA transcriptome. These studies were conducted under very diverse conditions, making comparisons between studies difficult. The mRNA transcriptional profile of intracellular *L. monocytogenes* 6 h after infection of Caco-2-epithelial cells was assessed by DNA microarray analysis (Joseph et al., 2006). In this study – as in every other that followed – the transcriptome of *L. monocytogenes* grown in rich media Brain Heart Infusion (BHI) served as reference. A similar approach was used to examine the transcriptome of *L. monocytogenes* when grown in murine macrophages P388D1 (Chatterjee et al., 2006). Here, the transcriptome of bacteria after 4 and 8 h post-infection as well as during intravascular growth was analyzed. Camejo et al. (2009) introduced in vivo work that shed light on the transcriptional profile of *L. monocytogenes* in murine spleens at 24, 48, and 72 h post-intravenous infection.

Despite the introduction of next generation sequencing (NGS) technologies, no study has addressed transcriptional changes in *L. monocytogenes* in detail. We now revisit the earlier microarray-based work (Chatterjee et al., 2006) complementing it with recent RNA-seq data (Wehner et al., 2014) which exclusively investigated non-coding RNAs of *L. monocytogenes* under intracellular and extracellular growth conditions.

Here, we report the transcriptional changes in *L. monocytogenes* during intracellular growth in murine macrophages using an approach that also detects alteration of non-coding RNA elements. Furthermore, we construct a framework for further analysis targeting intracellular survival of *L. monocytogenes*. Finally, we outline common transcriptional responses of *L. monocytogenes* during intracellular growth among all the diverse growth conditions investigated to date.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Culture Conditions**

All bacterial strains and plasmids used in this study are listed in Table 1. Wild type *L. monocytogenes* EGD-e serotype 1/2a (Glaser et al., 2001) and its isogenic deletion mutants Δlmo2316 or Δlmo1119 were cultivated under aerobic conditions in BHI broth (Difco, Becton Dickinson, Franklin Lakes, NJ, USA) or on BHI agar plates at 37°C. For RNA-seq experiments, *L. monocytogenes* EGD-e was grown in BHI broth overnight at 37°C with shaking at 180 rpm. Bacterial cultures were supplemented with fresh medium and grown until OD$_{600}$ reached 1.0.

*Escherichia coli* strain (DH10b) was grown in Luria-Bertani (LB) broth or on LB agar plates at 37°C. Cultures were supplemented with 300 μg/ml erythromycin (Sigma-Aldrich, St. Louis, MO, USA) for *E. coli* and 5 or 10 μg/ml for erythromycin for *L. monocytogenes* or 50 μg/ml ampicillin (Sigma-Aldrich, St. Louis, MO, USA) for *E. coli* and 200 μg/ml ampicillin for *L. monocytogenes*.

**Construction of Chromosomal Deletion Mutants Δlmo2316, Δlmo1119, and Δlmo1119/lmo2316**

Generation of lmo2316 or lmo1119 in frame deletion mutants was performed as previously described (Seifart Gomes et al., 2011). A detailed description is provided as Supplemental Text 1.

**RNA Isolation and RNA Sequencing**

Bacterial RNA isolation was carried out as described previously (Wehner et al., 2014). The quality of total RNA was assessed using Bioanalyzer 2100 Total RNA nano chip (Agilent, Santa Clara, CA, USA) and RNA concentration was measured using Qubit 2.0 fluorimeter (Invitrogen/Life Technologies, Carlsbad, CA, USA).

RQPCR was conducted under very diverse conditions, making comparisons between studies difficult. The mRNA transcriptional profile of intracellular *L. monocytogenes* 6 h after infection of Caco-2-epithelial cells was assessed by DNA microarray analysis (Joseph et al., 2006). In this study – as in every other that followed – the transcriptome of *L. monocytogenes* grown in rich media Brain Heart Infusion (BHI) served as reference. A similar approach was used to examine the transcriptome of *L. monocytogenes* when grown in murine macrophages P388D1 (Chatterjee et al., 2006). Here, the transcriptome of bacteria after 4 and 8 h post-infection as well as during intravascular growth was analyzed. Camejo et al. (2009) introduced in vivo work that shed light on the transcriptional profile of *L. monocytogenes* in murine spleens at 24, 48, and 72 h post-intravenous infection.

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**TABLE 1 |** Bacterial strains and plasmids used in this study.

| Strain or plasmid | Description | Reference |
|-------------------|-------------|-----------|
| *Listeria monocytogenes* EGD-e | Wild type | Glaser et al., 2001 |
| DH10b | Electrocompetent | Life technologies, Carlsbad, CA, USA |
| Δlmo1119 | lmo1119 isogenic deletion mutant | This study |
| Δlmo2316 | lmo2316 isogenic deletion mutant | This study |
| Δlmo1119/lmo2316 | lmo1119 and lmo2316 isogenic double deletion mutant | This study |
| pCR2.1-TOPO | single 3'-thymidine (T) overhangs for TA Cloning® 3.9 kb | Life technologies, Carlsbad, CA, USA |
| pAUL-A | Temperature sensitive shuttle vector 9.2 kb | Schäferkordt and Chakraborty, 1995 |
mRNA levels of \textit{lmo1119} and \textit{lmo2316} from both extra- and intracellularly grown bacteria were assessed using quantitative real-time-PCR (qRT-PCR) as previously described (Wehner et al., 2014). Each qRT-PCR was performed in triplicate (locus tag \_5' and locus tag \_3', Supplementary Table S1; 7900 HT Fast Real Time System, Applied Biosystems/Thermo Fisher Scientific, Waltham, MA, USA). Gene expression was normalized to 16S rRNA and relative expression of \textit{lmo1119} and \textit{lmo2316} was calculated using the mathematical method published by Pfaffl and Pfaffl (2001).

\textbf{In vitro Infection}

P388D1 murine macrophage were infected with \textit{L. monocytogenes} EGD-e and its isogenic deletion mutants as described previously (Mraheil et al., 2011).

\textbf{Statistical Analysis for qRT-PCR and Survival Assays Results}

All experimental work was repeated for a minimum of three times. Statistical analyses for qRT-PCR and survival assays were performed using R version 3.1.3. Data was log-transformed. Differential expression detected by qRT-PCR was tested by Student’s one-sample \(t\)-test. Differences in survival were analyzed using a linear regression model (Chambers and Hastie, 1992), where the amount of colony forming units (CFUs) was corrected by the inoculation amount as covariable. Pairwise comparisons were performed using the R package lsmeans version 2.17\textsuperscript{1} and multcomp (Hothorn et al., 2008). \(p < 0.05\) was considered significant.

\textbf{RESULTS}

We based our work on a recent study which included six sequencing runs (three runs for extracellular and three runs for intracellular growth conditions) performed with IonTorrent semiconductor sequencing technology (Wehner et al., 2014). This study investigated regulatory RNA, changes of mRNA expression were beyond the scope of the work. To extend and further back up this data set additional experiments were included using the reversible dye-terminator based sequencing approach of the Illumina MiSeq. These additional runs also facilitate detailed analysis of read mappings due to a higher amount of mapped reads and thus higher overall depth of coverage. In addition, it enables the evaluation whether transcriptomic changes occur platform-independently. Overall, pairwise comparison of normalized read counts provided no indication that variances in read counts between Illumina and Ion torrent runs exceeded variances of biological repeats among the Ion torrent runs (data not shown).

\textbf{Intracellular mRNA Transcriptome of \textit{L. monocytogenes}}

Comparing extracellular to intracellular growth conditions, our results show differential regulation for 656 genes (nearly 23\% of all genes). The genes with the most dramatic changes in expression are listed in Table 2.

\textsuperscript{1}http://CRAN.R-project.org/package=lsmeans
Moreover, Supplementary Table S2 provides a complete list of all genes that were differentially expressed under intracellular growth conditions. Of those 656 genes, 367 were predicted to be up- and 289 to be downregulated when grown in murine macrophages. These genes were further characterized using the previously introduced operon model (Toledo-Arana et al., 2009). Among the 656 differentially regulated genes, 386 are organized in operon structures while 270 are transcribed monocistronically. Further analysis of the differential expression pattern of these operons is given in Supplemental Text 2 as well as Supplementary Figure S1.

Concordance of RNA-seq Results with Microarray Data

When comparing our current results to the results of the previous microarray approach, we confirmed 55% of all differentially expressed genes found by the microarray-based approach. In both cases, an arbitrary threshold (p-value below 0.05) was applied to distinguish between differentially and not differentially expressed genes. A threshold of p < 0.1 in the current analysis increases the number to 64% of the previously found genes.

Five differentially regulated genes show opposing directions of regulation in both studies. These genes, one hypothetical gene as well as the metal transport operons lmo1423–1424 and lmo1848–1849, suggest that there have been minor experimental differences between both experimental conditions.

We then compared how the differentially expressed genes translate into alterations of functional pathways by applying the Clusters of Orthologous Groups of proteins (COG) classification. The number of differentially expressed genes is higher in the current RNA-seq study, however, the effects on the regulation of functional pathways is very similar (Figure 1).

Core Set of Genes Essential for Intracellular Growth

The investigated intracellular growth conditions vary widely among all published studies (Chatterjee et al., 2006; Joseph et al., 2006; Camejo et al., 2009; Toledo-Arana et al., 2009). We identified common transcriptional responses among these studies and our current work that are described in detail.

Virulence Genes are Upregulated during Intracellular Growth

Regardless whether epithelial cells, macrophages, blood, intestine, or spleens were infected, upregulation of the gene cluster lmo0201–0205 was reported under intracellular growth conditions. Genes encoded by this virulence gene cluster are actA (intracellular motility), metalloprotease (mpl), two phospholipases (plcA, plcB), and the pore forming toxin listeriolysin (hly). The virulence gene cluster is controlled by the positive regulatory factor PrfA. prfA was consistently upregulated during intracellular growth in all studies. In addition to the virulence cluster, PrfA-regulated expression has been reported for lmo0207 (orfZ), lmo0796, lmo0434 (iniB), lmo1786 (iniC), lmo2219 (prsA2), and lmo0838 (hpt). lmo0207 and lmo0206, which were significantly upregulated in all studies except one (Camejo et al., 2009), are genes of poorly understood function adjacent to the virulence cluster. lmo0434 (iniB) and lmo1786 (iniC) encoding internalins needed for invasion as well as lmo2219 (prsA2) encoding a predicted peptidyl-prolyl isomerase. The PrfA-regulated gene lmo0838 is involved in the transport of phosphorylated sugars. Apart from PrfA-regulated genes, the studies by Camejo, Chatterjee as well as our current study report upregulation of four VirR-regulated genes. Two of these genes are encoding hypothetical proteins (lmo0604 and lmo2177) and two are encoding ABC transporter (lmo2114 and lmo2115).

Stress-response Genes are Induced during Intracellular Growth

Upregulation of genes involved in stress response represents a second principal finding. This mechanism was found for L. monocytogenes grown in epithelial cells, macrophages,

| TABLE 2 | Except of differentially expressed genes under intracellular growth conditions compared to extracellular growth. |
|---------------|----------------------------------|
| Locus tag     | Gene                | Description                  | Regulation (intracellular) |
| lmo0205       | plcB                | Phospholipase C              | Up                        |
| lmo0203       | mpl                 | Zinc metalloproteinase       | Up                        |
| lmo2751       | lmo2751             | ABC transporter ATP-binding  | Up                        |
| lmo0204       | actA                | Actin-assembly inducing      | Up                        |
| lmo1786       | iniC                | Internalin C                 | Up                        |
| lmo0207       | lmo0207             | Hypothetical protein         | Up                        |
| lmo0838       | hpt                 | Sugar:phosphate antipporter  | Up                        |
| lmo0608       | lmo0608             | ABC transporter ATP-binding  | Up                        |
| lmo2828       | lmo2828             | Hypothetical protein         | Up                        |
| lmo0206       | lmo0206             | Hypothetical protein         | Up                        |
| lmo0751       | lmo0751             | Hypothetical protein         | Up                        |
| lmo2752       | lmo2752             | ABC transporter ATP-binding  | Up                        |
| lmo0576       | lmo0576             | Hypothetical protein         | Up                        |
| lmo0955       | lmo0955             | Hypothetical protein         | Up                        |
| lmo0514       | lmo0514             | Internalin                   | Up                        |
| lmo0749       | lmo0749             | Hypothetical protein         | Up                        |
| lmo2102       | lmo2102             | Glutamate amidotransferase   | Down                      |
| lmo0954       | lmo0954             | Hypothetical protein         | Up                        |
| lmo0748       | lmo0748             | Hypothetical protein         | Up                        |
| lmo0750       | lmo0750             | Hypothetical protein         | Up                        |
| lmo2827       | lmo2827             | MarR family transcriptional  | Regulator                 |
| lmo0607       | lmo0607             | ABC transporter ATP-binding  | Up                        |
| lmo0201       | plcA                | Phosphatidylinositol-specific| Up                        |
spleenocytes, and bacteria grown in the intestine (Chatterjee et al., 2006; Joseph et al., 2006; Camejo et al., 2009; Toledo-Arana et al., 2009) Specifically, the upregulation of lmo1473 (dnaK), lmo1474 (grpE), and lmo2068 (groEL) belonging to heat shock molecular chaperons (class I) as well as lmo2206 (clpB) which is an ATP-dependent protease (class III), was found in all studies. In addition to the induced transcription of lmo1473 and lmo1474, upregulation of lmo1471 (prmA), lmo1472 (dnaJ) and lmo1475 (hrcA), being part of the same operon, was found by our study as well as by the previous microarray-approach. Furthermore, our study suggests a broader transcriptional upregulation for class III heat shock genes. Transcripts of the whole major heat shock class III operon consisting of lmo0229 to lmo0232 were significantly increased. Especially, lmo0232 (clpC) was significantly upregulated in all studies except the one using epithelial cells as host. Similarly, the caseinolytic protease proteins lmo0997 (clpE) was upregulated in splenocytes and macrophages.

**Metabolic Pathways are Differentially Regulated during Intracellular Growth**

The last common finding among all studies concerns the intracellular transcriptional upregulation for lmo1538. lmo1538 encodes a glycerol kinase that is required for intracellular growth. Regulation of glycolysis under intracellular growth conditions was also addressed by the previous studies. Central genes of glycolysis were upregulated when L. monocytogenes was grown in splenocytes (Camejo et al., 2009), the studies in epithelial cells and macrophages found downregulation (Chatterjee et al., 2006; Joseph et al., 2006). For instance, it was observed that the operon lmo2455–2460, encoding five essential enzymes for the terminal glycolysis steps, has been downregulated.

**Novel Findings of this Study**

Additional metabolic changes extending previous findings involve the transport of sugar and sugar phosphates. Our results provide evidence for the downregulation of mannose transporter subunits lmo0096–0098 as well as lmo1389–1390, genes involved in sugar transport. In contrast, upregulation was observed for lmo0736–0739, contributing to sugar transport and processing of phosphorylated ribulose, and an entire operon (lmo2733–2736), encoding fructose transporter, sugar hydrolyase and sucrose phosphorylase. Remarkably, the operon lmo2670–2672 was also uniformly upregulated during intracellular growth, including lmo2671, a hypothetical protein that has similarity to a lactoylglutathione lyase. Genes required for phosphate transport were also differentially expressed. One of those loci consists of the genes lmo2494–2499, encoding proteins for phosphate uptake and transport. In addition, operon lmo2248 (a putative phosphate transport regulator)/lmo2249 (a low-affinity inorganic phosphate transporter) was downregulated during intracellular growth. Finally, several genes involved in DNA repair, e.g., recA (lmo1398) and the excinucleases lmo2050, lmo2488 (uvrA)/lmo2489 (uvrB) were differentially expressed during intracellular growth conditions.

**Antisense Transcription is Altered during Intracellular Growth**

A benefit of strand-specific RNA-seq studies is the ability to detect antisense transcripts as well as sense transcripts. We investigated changes in antisense expression...
between intra- and extracellular growth conditions. These analyses were not possible with previous microarray based approaches.

For many of the genes showing dramatic differential antisense expression during intracellular growth, adjacent divergent genes displayed drastic changes in sense expression. Examples are antisense transcripts against \emph{lmo0208–0209} and \emph{lmo0198–0199} both flanking the virulence gene cluster. Similarly, antisense transcripts of \emph{lmo0839} (neighboring the divergently oriented \emph{hpt}) and of the sugar transferase gene \emph{lmo0497} (adjacent a highly upregulated SOS response gene \emph{lmo0496}), were highly induced.

We also detect strong antisense upregulation for \emph{lmo1705} that was previously reported only as marginal note in the supplement (Wehner et al., 2014). With the additional sequencing runs that provided a higher read coverage, we obtained a distinct picture. Sense transcripts are slightly downregulated during intracellular growth while antisense transcripts are upregulated. When analyzing the read pattern (Figure 2) hardly any read was spanning the intergenic region between \emph{lmo1705} and \emph{lmo1706}. Hence, the increase of antisense reads could not be attributed to an erroneous lack of transcription termination of the adjacent \emph{lmo1706}. Moreover, a high coverage was found for the intergenic region between \emph{lmo1705} and \emph{lmo1704}. Operon \emph{lmo1702–1704} appears to have an additional 5′ UTR transcription start site, which overlaps \emph{lmo1705}. The complete list with genes for which putative changes in antisense expression were found is provided in Supplementary Table S3.

Regulation of the A118-like Prophage Genes in Intracellular Conditions

In our study, we found increased expression of a cassette of A118-like prophage genes (\emph{lmo2271–2332}) under intracellular growth conditions. In contrast, only few genes were predicted to be upregulated by the previous studies.

In analogy to the bacteriophage A118 described previously (Loesner et al., 2000), three organizational clusters, namely ‘late genes,’ ‘early genes,’ and ‘lysisogeny control,’ can be distinguished in the phage genome. The expression among these clusters differs widely (Figure 3).

The late genes (\emph{lmo2278–2301}) comprise genes coding for proteins required for host cell lysis, structural and assembly proteins as well as proteins for DNA packaging. All these genes are poorly expressed under any growth condition. Evidence for upregulation under intracellular growth condition was only found for \emph{lmo2299} most likely encoding a portal protein. Beside a generally low expression, antisense transcripts were detected for all genes of this locus expressed under both investigated conditions. For the lysis factors holin and \emph{lysA}, antisense transcripts were almost as frequent as sense transcripts and for three minor capsid proteins (\emph{lmo2292–2294}) antisense transcripts even exceeded the amount of sense transcripts.

The early genes (\emph{lmo2302–2328}), encoding for products of replication, recombination, and modification of phage DNA, showed an identical expression pattern compared to the late genes during extracellular growth. During intracellular growth, gene expression of early genes was strongly induced with 23 genes significantly upregulated.

The third cluster involved in lysogeny control (\emph{lmo2271–2277} and \emph{lmo2329–2332}) flanks both insertion sites and contains several putative transcriptional factors as well as the integrase. On a transcriptional level, all of these genes were well-expressed under both intra- and extracellular growth conditions.

Of particular interest are the genes \emph{lmo2328} and \emph{lmo2329}. Both of these adjacent and divergently oriented genes possess a helix-turn-helix motif common for transcriptional regulators. This constellation is a component of several bacteriophage genomes. In \emph{E. coli} phage \emph{λ}, homologs of these genes are involved in the switch between lysogeny and lytic behavior (Oppenheim et al., 2005). In our study, \emph{lmo2329} is highly expressed under both conditions, but \emph{lmo2328} is exclusively expressed under intracellular growth conditions.

To assess whether phage excision takes place thereby restoring functionality of the previously split competence activator \emph{comK} (\emph{lmo2270} and \emph{lmo2333}), we mapped the sequencing reads against \emph{L. monocytogenes} EGD (downloaded from NCBI NC_022568) using relaxed mapping conditions. \emph{L. monocytogenes} EGD is highly related to \emph{L. monocytogenes} EGD-e but does not have the A118-like prophage insertion. The read mapping (Figure 4) highlights that not even a single read is spanning the phage insertion site under intracellular growth conditions. Therefore, no evidence for excision restoring \emph{comK} was detected. In addition, no significant difference was found between intra- and extracellular growth for the gene expression of competence cluster \emph{comF} and \emph{comG} as well as the genes \emph{comEA} and \emph{comEB}. Only expression of \emph{comEC} was increased under intracellular conditions (Supplementary Figure S2).

Within the phage region, \emph{lmo2316} encoding a putative site-specific adenine methylase (based on KEGG database prediction) was highly upregulated during intracellular growth. Methylases are known to contribute to pathogenicity and intracellular survival. \emph{lmo2316} was further characterized and the bacterial methyltransferase \emph{lmo1119}, which is located elsewhere in the bacterial genome and is predicted to have similar functions, was included in the analysis as phage-independent control. Of note, the gene locus of \emph{lmo1119} indicates significant difference in its GC content compared to the whole genome sequence (Supplementary Figure S3).

While expression of the phage gene \emph{lmo2316} was strongly induced during intracellular growth, no difference in \emph{lmo1119} expression was detected under intracellular growth conditions. However, \emph{lmo1119} was well-expressed under both growth conditions whereas \emph{lmo2316} is hardly expressed at all under extracellular growth conditions.

Experimental Validation of \emph{lmo2316} and \emph{lmo1119}

\textit{In vitro} and \textit{In vivo}

We used qRT-PCR analysis to confirm the transcriptional changes of \emph{lmo1119} and \emph{lmo2316} indicated by RNA-seq. As predicted, \emph{lmo2316} was significantly upregulated (\emph{p} = 0.0014) and \emph{lmo1119} showed a trend toward downregulation during intracellular growth (\emph{p} = 0.1091; Figure 5A).

In order to assess the effect of the phage methylase on intracellular growth properties of \emph{L. monocytogenes}, we generated
a knockout mutant and investigated its ability to survive in P388D1 murine macrophages compared with the wild type strain (L. monocytogenes EGD-e). lmo1119 single and lmo1119/lmo2316 double mutants were included as controls in this experiment. Δlmo1119 and Δlmo1119/lmo2316 were significantly impaired in their intracellular survival compared to L. monocytogenes EGD-e. In contrast, Δlmo2316 survived similar to L. monocytogenes EGD-e. Similarly, no additive effect of Δlmo1119/lmo2316 was observed in comparison to Δlmo1119 single mutant (Figure 5B, Supplementary Table S4). Growth in BHI was not altered in the knockout strains in comparison to wild type L. monocytogenes EGD-e (data not shown).
In conclusion, even though *lmo2316* was significantly induced during intracellular growth, no contribution to intracellular survival of *L. monocytogenes* grown in murine macrophages was found.

**DISCUSSION**

This study reveals significant differences in the transcriptional response of *L. monocytogenes* for 23% of the genes when grown
in murine macrophages compared to growth in BHI. This exceeds the microarray findings where 17% were differentially regulated (Chatterjee et al., 2006). Given the enormous differences between growth in rich media such as BHI and the cytosolic growth conditions including induction of stress responses, 23% seems reasonable if not rather low. The direct comparison between results of both studies show a high degree of similarity particularly regarding methodological differences.

This work reveals several transcriptional adaptations to intracellular growth conditions that appear to be universal, as they have been reported in previous studies after infection of epithelial cells, macrophages, and splenocytes (Chatterjee et al., 2006; Joseph et al., 2006; Camejo et al., 2009). Transcriptional upregulation of prfA and PrfA-dependent genes is one of those strategies. The majority of these upregulated genes are organized in the virulence gene cluster (lmo0201–0205). The
phospholipases \( plcA,\) \( plcB,\) and listeriolysin are crucial for phagosomal escape. The metalloprotease \( Mpl\) is involved in maturation of \( plcB\) and the product of \( actA\) facilitates actin assembly beneficial for cell-to-cell spread of \textit{Listeria} (Cossart, 2011). Apart from this locus, two internalin genes, \textit{prsA2} and \textit{hpt} were upregulated. While Internalin B (\textit{inlB}) is required for cell entry (Gaillard et al., 1991), internalin C (\textit{inlC}) is involved in cell-to-cell (Engelbrecht et al., 1996; Rajabian et al., 2009) spread and interferes with the innate immune response of the host (Gouin et al., 2010). The chaperonin \textit{lmo2219} (\textit{prsA2}) is thought to contribute to virulence, e.g., by promoting the stability of listeriolysin (Alonzo et al., 2009; Cahoon and Freitag, 2015). \textit{Hpt} is involved in the transport of sugar-phosphates and is required for efficient intracellular proliferation (Chico-Calero et al., 2002). Thus, a predominant carbon source other than glucose has been suggested during intracellular growth (Joseph et al., 2006). Our sequencing results show differential regulation of several operons involved in sugar transport. Among those, the mannose/glucose transport operon (\textit{lmo0096–0098}) was downregulated. Expression of this operon has recently been demonstrated to play a role in glucose mediated virulence gene repression (Zebré et al., 2015).

Of note, \textit{lmo1538}, encoding a glycerol kinase, was found to be upregulated after infection of epithelial cells, macrophages and splenocytes (Chatterjee et al., 2006; Joseph et al., 2006; Camejo et al., 2009). Therefore, glycerol represents an alternative carbon source (Joseph et al., 2008; Grubmüller et al., 2014). In this context, the downregulation of central genes for glycolysis was discussed. While this downregulation occurred for \textit{L. monocytogenes} infecting epithelial cells and macrophages (Chatterjee et al., 2006; Joseph et al., 2006; Camejo et al., 2014), \textit{L. monocytogenes} grown in spleens showed an opposite trend (Camejo et al., 2009). Our study confirmed this downregulation of several glycolysis genes and showed upregulation of an operon including a predicted glycolyolase (\textit{lmo2671}). Thus, it is interesting to figure out whether the glycolyolase pathway, also known to function as an offshoot to glycolysis, is involved in the adaptation to intracellular growth conditions.

Another universal transcriptional response is the upregulation of genes involved in stress response. Maintenance of proper protein folding during a stress response is achieved by chaperons and ATP-dependent proteases that prevent the accumulation of misfolded proteins. Our results showed upregulation of the \textit{dnaK}-operon and caseinolytic proteases genes (\textit{clpB}, \textit{clpE}, and \textit{clpC}). The importance for intracellular survival of all these caseinolytic proteases has been demonstrated (Rouquette et al., 1996; Nair et al., 1999; Chastanet et al., 2004).

In our study, we address both intracellular sense and antisense regulation in \textit{L. monocytogenes} for the first time. We provide evidence for regulation of several genes under intracellular growth conditions. Antisense regulation in \textit{L. monocytogenes} has not been investigated thoroughly although it might be of utmost importance for translation (Melin and Cossart, 2012; Behrens et al., 2014; Schultze et al., 2014; Wehner et al., 2014). We found antisense regulation targeting \textit{lmo1705}, a deoxyguanosine kinase/deoxyadenosine kinase. Read mappings of this region prompt a linkage to the transcription of the adjacent divergent operon \textit{lmo1702–1704}. Therefore, this locus might represent a putative excludon activated under intracellular growth conditions.

In addition to the bacterial transcriptional profile, we also provide the transcriptional response of a bacteriophage to intracellular growth conditions. During the last few years, bacteriophages and their response to environmental changes were increasingly investigated as they represent a mean to counteract food-borne pathogens (Denes and Wiedmann, 2014). The greatest transcriptional changes in the prophage region occurred in the ‘early genes.’ The function of ‘early genes’ includes DNA replication, modification, recombination, and gene expression modification. Among the upregulated genes, we focused on a site-specific DNA methylase \textit{lmo2316}. It is well-known that DNA methylation is crucial for epigenetic control of gene expression in bacteria. Phenomena such as timing of DNA replication, DNA repair, protection against foreign bacteriophage DNA, timing of transposition, pilus expression and conjugal plasmid transfer are sensitive to the methylation states of specific DNA regions (Sánchez-Romero et al., 2015). DNA methylases are also involved in virulence (Heusipp et al., 2007). Yet, intracellular survival in macrophages was not affected by the deletion of \textit{lmo2316}. Recent studies outline the importance of methylases in the evolutionary arms race between bacteria and bacteriophages (Labrie et al., 2010; Goldfarb et al., 2015). Given that, it is more likely that this particular DNA methylase modifies newly synthesized phage DNA to evade bacterial restriction modification (R–M) systems. Several R–M systems have been previously reported for \textit{L. monocytogenes} epidemic clone I and II (Sau3AI-like, LmoH7, LmoJ2, and LmoJ3) which were involved in temperature dependent phage resistance (Yildirim et al., 2010; Kim et al., 2012; Lee et al., 2012). Impaired growth of \textit{Δlmo1119} in macrophages might be explained by the role of \textit{lmo1119} in maintenance of the bacteria-specific DNA methylation pattern. Thus, a knockout of this gene can be detrimental due to its own R-M system. Of note, both gene loci (\textit{lmo2316} and \textit{lmo1119}) indicate an unusually low GC content. \textit{lmo2316} is located in the well-known A18-like prophage locus whereas \textit{lmo1119} is a downstream neighbor of TN916. The latter chromosomal locus was previously designated as “hypervariable hotspot 8” where LIPI-3 is inserted in lineage I strains \textit{L. monocytogenes} (Glaser et al., 2001; Cotter et al., 2008; Kuenne et al., 2013). This suggests that both gene regions originated by horizontal gene transfer via transduction and/or transposition.

Whether the temperate phage remains in lysogenic state or switches into the lytic cycle and thereby eventually lyses its host, is of crucial importance for survival of the bacteria. For the \textit{E. coli} phage \( \lambda \), the two adjacent and divergently oriented transcriptional regulators \textit{cl} and \textit{cro} are crucial for the ‘lysis-lysogeny decision.’ Gene products of \textit{cl} repress the ‘early phage’ genes including \textit{cro}. Gene products of \textit{cro} in turn repress transcription of \textit{cl} directly as well as indirectly by decreasing transcription of an activator of \textit{cl}. Currently, the indirect route is thought to have the major effect (Dodd et al., 2005; Oppenheim et al., 2005).
Homologs of $cI$ and $cro$ have been described for *Lactococcus lactis* phage (Kodaira et al., 1997) or the *L. monocytogenes* A118 phage (Loessner et al., 2000). Similar to A118 phage, the A118-like bacteriophage in *L. monocytogenes* EGD-e contains the genes lmo2328 and lmo2329 exhibiting similarity to $cI$ and $cro$, respectively.

However, RNA-seq data contradicts the proposed mechanism of mutual repression of lmo2328 and lmo2329 for the A118-like prophage. During intracellular growth, the homologs to both $cro$ and $cI$ were highly expressed. Hence, the mechanism of switching between lysogeny and lytic state known from *E. coli* phage $\lambda$ is probably not completely applicable in *L. monocytogenes* EGD-e.

Yet, upregulation of lmo1398 (recA) during intracellular growth might explain the expression of the ‘early genes’ including lmo2328 (homolog to $cro$) despite the presence of lmo2329 (homolog to the inhibitor of early genes $cI$) transcripts. In *E. coli*, RecA supports cleavage of $cI$ and thereby derepresses the ‘early genes.’

Of note, the ‘late genes’ are only poorly expressed under both growth conditions. The discovery of antisense transcripts in this region indicates another inhibitory mechanism.

Our study also provided further insights concerning the excision of the A118-like prophage of *L. monocytogenes* EGD-e when grown in macrophages. In *L. monocytogenes* strain 10403S a comparable phage insertion was investigated in murine bone-marrow derived macrophages under intracellular growth conditions (Rabinovich et al., 2012). This study demonstrated that prophage excision takes place under intracellular growth conditions thereby restoring functionality of the previously split competence activator comK (Rabinovich et al., 2012). We found no evidence for such an excision of the prophage genome. This is corroborated by unaltered transcript expression of the phage integrase under both growth conditions.

Moreover, the increased gene expression of all three competence gene clusters (comE, comG, and comK) in *L. monocytogenes* 10403S due to restoration of comK (Rabinovich et al., 2012) was not confirmed for *L. monocytogenes* EGD-e. In contrast, our transcriptional profile showed no intracellular upregulation of the clusters comG and comK as well as no changes in comEA and comEB expression. Only comEC expression was intracellularly upregulated in our study. Interestingly, only comG and comEC were shown to be beneficial for intracellular survival probably by alleviating the phagolysosomal escape (Rabinovich et al., 2012). We propose that in *L. monocytogenes* EGD-e, comEC can be alternatively activated bypassing the global competence activator comK.

**CONCLUSION**

Here, we present the first intracellular mRNA transcriptome for *L. monocytogenes* obtained using an NGS approach. RNA-seq-based studies have the power to study both coding and non-coding RNA phenomena. Our data is supported by previous microarray studies. In summary, we provide a base for future work targeting intracellular survival of *L. monocytogenes* covering both sense and antisense transcript changes. By comparing mRNA transcriptome data from different studies and different stages of listerial infection (epithelial cells, macrophages, and spleen as target organ), we identified a common set of genes always upregulated during intracellular growth. Furthermore, we provide the transcriptional profile of a temperate A118-like phage thereby offering novel insights in phage control and the intertwined coexisting between bacteriophage and bacteria. Taken together, this work expands the view on the transcriptional changes arising from intracellular growth conditions.

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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.2015.01199

**FIGURE S1 | Coverage plot of all operons for which differential expression patterns were in discord with the predicted operon model** (Toledo-Arana et al., 2009). SigB operon (lmo0887-0896) shown in (A), lmo1416-1420 in (B) and lmo2463-2465 given in (C).

**FIGURE S2 | Read mappings of all competence clusters.** The competence cluster comG and comF are poorly expressed. Only comEC (lmo1482) was significantly upregulated during intracellular growth.

**FIGURE S3 | Visualization of the chromosomal regions of lmo1119 (A) and lmo2316 (B) using GECO** (Kuenne et al., 2007). The line below the illustrated genes indicates the deviation from the average GC content. Values greater than zero are highlighted by brown colour while those below zero are blue.

**Comparison of the A118-like prophage region between the *Listeria monocytogenes* strains EGD-e, 10403S and EGD.** Genes are colored according to similarity on protein level. Core genes are indicated in black.

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