Universal Stress Proteins Are Important for Oxidative and Acid Stress Resistance and Growth of *Listeria monocytogenes* EGD-e *In Vitro* and *In Vivo*

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**Abstract**

**Background:** Pathogenic bacteria maintain a multifaceted apparatus to resist damage caused by external stimuli. As part of this, the universal stress protein A (UspA) and its homologues, initially discovered in *Escherichia coli* K-12 were shown to possess an important role in stress resistance and growth in several bacterial species.

**Methods and Findings:** We conducted a study to assess the role of three homologous proteins containing the UspA domain in the facultative intracellular human pathogen *Listeria monocytogenes* under different stress conditions. The growth properties of three UspA deletion mutants (*Δlmo0515, Δlmo1580* and *Δlmo2673*) were examined either following challenge with a sublethal concentration of hydrogen peroxide or under acidic conditions. We also examined their ability for intracellular survival within murine macrophages. Virulence and growth of *usp* mutants were further characterized in invertebrate and vertebrate infection models. Tolerance to acidic stress was clearly reduced in *Δlmo1580* and *Δlmo0515*, while oxidative stress dramatically diminished growth in all mutants. Survival within macrophages was significantly decreased in *Δlmo1580* and *Δlmo2673* as compared to the wild-type strain. Viability of infected *Galleria mellonella larvae* was markedly higher when injected with *Δlmo1580* or *Δlmo2673* as compared to wild-type strain inoculation, indicating impaired virulence of bacteria lacking these *usp* genes. Finally, we observed severely restricted growth of all chromosomal deletion mutants in mice livers and spleens as compared to the load of wild-type bacteria following infection.

**Conclusion:** This work provides distinct evidence that universal stress proteins are strongly involved in listerial stress response and survival under both *in vitro* and *in vivo* growth conditions.

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**Introduction**

Universal stress proteins (Usps) comprise a group of proteins induced by different stress conditions and are found in numerous prokaryotic as well as eukaryotic organisms [1,2]. Among these, universal stress protein A (UspA) of *Escherichia coli* K-12 is best characterized and found to be highly expressed in response to heat, substrate starvation, exposure to antimicrobial agents and oxidative stress [1].

Subsequently, additional Usps have been described for *E. coli* K-12 and several other bacterial species, including *Haemophilus influenzae* [3,4], *Mycobacterium smegmatis* [5], *Mycobacterium tuberculosis* [2,6], *Pseudomonas aeruginosa* [7–9], *Porphyromonas gingivalis* [10], *Shigella sonnei* [11], *Salmonella typhimurium* [12–14] and *Lactobacillus plantarum* [15,16].

The majority of *usp* genes are monocistrionically expressed, and different transcription factors, such as σ32, σ70 and σ5 promote transcription of Usps [1].

The significance of Usps in the model pathogen *Listeria monocytogenes* is presently unknown. *L. monocytogenes* is a ubiquitously occurring gram-positive, facultative intracellular bacterium that causes food-borne infections, which mainly affect pregnant women, newborns, elderly and immunocompromised patients [17,18]. *L. monocytogenes* possesses the remarkable ability to grow under a wide range of temperatures, pH conditions and high osmolality, allowing the pathogen to survive in nature, food preservation methods as well as in infected host cells [18].

Here we present first evidence that UspA domain containing stress proteins are of importance for *L. monocytogenes* to survive under different stress conditions both *in vitro* and *in vivo*.

**Materials and Methods**

**Bacterial strains, plasmids and culture conditions**

All bacterial strains and plasmids used in this study are listed in Table 1.
The wild type strain *L. monocytogenes* EGD-e serotype 1/2a [19] and its isogenic *usp* deletion mutants or complemented strains were cultivated aerobiically in Brain Heart Infusion medium (BHI; Difco) at 37°C or on BHI agar plates at 37°C.

E. coli strain (DH10B<sup>R</sup>) was grown in Luria-Bertani broth or LB agar plates at 37°C. When required, antibiotics were added to the following concentrations: erythromycin (Sigma), 300 μg/ml for *E. coli* and 5 or 10 μg/ml for *Listeria*; ampicillin (Sigma), 50 μg/ml for *E. coli* and 200 μg/ml for *Listeria*.

### Construction of chromosomal *usp* deletion mutants

Generation of the *usp* in frame deletion mutants was done as previously described [20–22]. Briefly, the flanking regions of each *usp* gene were amplified by PCR (TA polymerase; Fermentas) using primer pairs 1 and 2b for the 5′ gene were amplified by PCR (Table 2), respectively. The resulting PCR products were fused to each other by using primers 1 and 4 in a second PCR reaction with Taq polymerase; Fermentas) and the product was cloned into pCR2.1-TOPO<sup>®</sup>.

For the generation of Δimo0515 and Δimo2673 the pCR2.1-TOPO<sup>®</sup> vector containing the flanking region of the respective gene was digested with BamH<sub>I</sub>, Xho<sub>I</sub> and Not<sub>I</sub> (Fermentas) and inserted into pAUL-A [21], that was previously digested with BamH<sub>I</sub> and SalI (Fermentas). The pCR2.1-TOPO<sup>®</sup> vector containing the flanking region of Δimo1580 was digested with XhoI and SalI (Fermentas) and ligated with pAUL-A, also previously digested with the same restriction endonucleases.

We designated resulting pAUL-A vectors containing the flanking regions of the respective *usp* genes, pCS1 for Δimo0515, pCS2 for Δimo1580 and pCS3 for Δimo2673. These vectors were transformed into *E. coli* and were isolated, sequenced and subsequently electroporated into *L. monocytogenes* wild-type strain. Gene replacement was performed as previously described by Schaeferkordt et al. [21,22]. To create a triple mutant (Δimo0515Δimo1580Δimo2673) for all three *usp* genes, gene replacement were carried out as described above starting with the electroporation of pCS2 into Δimo0515. Finally, pCS3 was electroporated into the resulting double mutant to generate the isogenic triple mutant (Δimo0515Δimo1580Δimo2673). The chromosomal deletion was confirmed by DNA sequencing of PCR products using primer 7 and 8 (Table 2).

### Table 1. Bacterial strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. monocytogenes</em> EGD-e</td>
<td>wild-type</td>
<td>[19]</td>
</tr>
<tr>
<td>DH10B&lt;sup&gt;R&lt;/sup&gt;</td>
<td>electrocomptent</td>
<td>Life technologies</td>
</tr>
<tr>
<td>Δimo0515</td>
<td>lmo0515 deletion (379 nt) strain of EGD-e</td>
<td>This study</td>
</tr>
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<td>Δimo1580</td>
<td>lmo1580 deletion (409 nt) strain of EGD-e</td>
<td>This study</td>
</tr>
<tr>
<td>Δimo2673</td>
<td>lmo2673 deletion (471 nt) strain of EGD-e</td>
<td>This study</td>
</tr>
<tr>
<td>Δimo0515Δimo1580Δimo2673</td>
<td>lmo0515, lmo1580 and lmo2673 deletion strain of EGD-e</td>
<td>This study</td>
</tr>
<tr>
<td>Δimo0515+pPL2imo0515</td>
<td>complementation of lmo0515 deletion strain of EGD-e</td>
<td>This study</td>
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<td>complementation of lmo1580 deletion strain of EGD-e</td>
<td>This study</td>
</tr>
<tr>
<td>Δimo2673+pPL2imo2673</td>
<td>complementation of lmo2673 deletion strain of EGD-e</td>
<td>This study</td>
</tr>
<tr>
<td>pPL2</td>
<td>site specific phage integration vector</td>
<td>[23]</td>
</tr>
<tr>
<td>pCR2.1-TOPO&lt;sup&gt;®&lt;/sup&gt;</td>
<td>single 3′-thymidine (T) overhangs for TA Cloning&lt;sup&gt;®&lt;/sup&gt; 3.9 kb</td>
<td>Life technologies</td>
</tr>
<tr>
<td>pAUL-A</td>
<td>temperature sensitive shuttle vector 9.2 kb</td>
<td>[21]</td>
</tr>
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</table>

### Table 2. Primers used in this study.

<table>
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<td>lmo0515-1</td>
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<tr>
<td>lmo0515-2b</td>
<td>AAGTGTCCGTCGACATGCCGC</td>
</tr>
<tr>
<td>lmo0515-3b</td>
<td>CTTGACACGACACATTTAAGTCA</td>
</tr>
<tr>
<td>lmo1580-4</td>
<td>TTTGGGCTGCTACCTACGC</td>
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<tr>
<td>lmo1580-5</td>
<td>ATGGTTGGAATCTGTGCA</td>
</tr>
<tr>
<td>lmo1580-6</td>
<td>CGACATGCAGACTGTGATC</td>
</tr>
<tr>
<td>lmo1580-7</td>
<td>ACCCAATTGGTTGATCGCATG</td>
</tr>
<tr>
<td>lmo1580-8</td>
<td>GGGTCATTGCCACCCTATT</td>
</tr>
<tr>
<td>lmo1580-9</td>
<td>GTTTTGGTACCCCTTACCC</td>
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<td>lmo2673-1</td>
<td>TAAAGGCTGACTCTGGTACA</td>
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<tr>
<td>lmo2673-2b</td>
<td>TATACATGATTTGATCTGCAATCC</td>
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<tr>
<td>lmo2673-3b</td>
<td>GTGATACAAATGCTGATAGGAAGTAGT</td>
</tr>
<tr>
<td>lmo2673-4</td>
<td>CCAACAGGCGCAACAGTACCC</td>
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<tr>
<td>lmo2673-5</td>
<td>AAAAGACCTTCCTTCTTCTT</td>
</tr>
<tr>
<td>lmo2673-6</td>
<td>TTTGCTCTCCGATCGT</td>
</tr>
<tr>
<td>lmo2673-7</td>
<td>AGCAGCGTCGCACTAGTTGG</td>
</tr>
<tr>
<td>lmo2673-8</td>
<td>GTATCTGCCGAGTCACT</td>
</tr>
<tr>
<td>lmo0515-forward</td>
<td>GATGTTGCAAGAAACCAGAAA</td>
</tr>
<tr>
<td>lmo0515-reverse</td>
<td>GCTTTTCTTCTAAGTCGCCAT</td>
</tr>
<tr>
<td>lmo1580-forward</td>
<td>GCAGTGTAGGATCAAGAAAGA</td>
</tr>
<tr>
<td>lmo1580-reverse</td>
<td>TTATCCGACATGGTTCGTC</td>
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<tr>
<td>lmo2673-forward</td>
<td>ATACACGCGAAATTTGGTACAC</td>
</tr>
<tr>
<td>lmo2673-reverse</td>
<td>CCAAGGTTTGGTTGAGGAA</td>
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</table>

doi:10.1371/journal.pone.0024965.t001

**Table 1.** Bacterial strains and plasmids used in this study.

**Table 2.** Primers used in this study.
The complementation of the Δlmo0515, Δlmo1580 and Δlmo2673 deletion mutants was carried out using the L. monocytogenes site specific phage integration vector pPL2 [23]. PCR products were generated using primer pairs 5 and 6 with Taq polymerase. The product was cloned into pCR 2.1-TOPO®. The pCR 2.1-TOPO® vector containing the wp gene region of lmo0515 or lmo2673 was digested with BamHI and XhoI (Fermentas) and for lmo1580 with XhoI and SacI (Fermentas), respectively. The insert was ligated with pPL2, also previously digested with the same restriction endonucleases. Primer sequences used for the complementation of the deleted genes are listed in table 2.

Acid stress assay

Single colonies of the wild type L. monocytogenes and its wp deletion mutants grown on BHI agar plates were used to inoculate 100 ml Erlenmeyer flasks containing 10 ml of BHI broth, followed by overnight incubation at 37°C with shaking (180 rpm, Unirtron Infors). The overnight cultures were then subcultured (1:50) in 40 ml of BHI and grown to an optical density at 600 nm (OD600) of 0.4 prior to our experiments. Then each culture was divided into two aliquots, 20 ml each and cells were harvested by centrifugation at 6,000 x g for 15 min. at 37°C. Furthermore the supernatant was removed and cells were resuspended in a BHI broth that was acidified with a 6 N HCl solution (Sigma) to pH 2.5. These tubes were incubated with shaking (180 rpm) at 37°C. At time point zero (t = 0) and after 10, 20 and 30 minutes intervals samples were taken from each culture, serially digested with H2O2, as mentioned before. The colony forming units (CFU) were counted after centrifugation at 37°C. The percentage of survivors was acquired and analyzed with the SDS 2.3 and RQ-Manager 1.2, respectively.

Hydrogen peroxide sensitivity assay

BHI broth was used to dilute a 3% hydrogen peroxide (H2O2) solution (Ottmar Fischer, Germany) to obtain 0.045% H2O2 final concentration.

Overnight cultures in 10 ml BHI broth (in 100 ml Erlenmeyer flasks) were prepared and were diluted 1:50 in 20 ml fresh BHI broth or in 20 ml of different fresh BHI supplemented with 0.045% H2O2 (in 100 ml Erlenmeyer flasks) as mentioned before. The optical density (OD600) of the bacterial culture was measured at the indicated times after incubation at 37°C (180 rpm, Unirtron, Infors).

Macrophage survival assay

P388D1 murine macrophages, cultured in 24-well plates in RPMI (Life Technologies) supplemented with 10% fetal calf serum (FCS) and 1% nonessential amino acids (NEAs), were infected with 1 x 10⁷ bacteria to obtain an MOI of 10. After 30 min. RPMI was removed, and cells were washed twice with 1 x PBS and incubated for 1 h in RPMI medium containing 50 µg/ml gentamicin. Macrophages were then washed three times with 1 x PBS and lysed with ice-cold 0.2% Triton X-100 in H2O. The released bacteria were plated on BHI agar plates in appropriate dilutions and quantified after overnight incubation at 37°C.

For the determination of macrophage survival rates the CFU numbers were calculated. The wild-type factor was set to 1 and depending on the CFU numbers of the mutant inoculums their factor was <1 or >1. For the calculation of the survival in % we set the CFU number of wild-type to 100% and percentage survival of the mutants was set in relation to wild-type.

cDNA synthesis and quantitative Real-Time Polymerase Chain Reaction

Messenger RNA levels of lmo0515, lmo1580 and lmo2673 isolated from extracellular grown bacteria in BHI and intracellular replicating bacteria in murine macrophages as previously described [24] were assessed in L. monocytogenes wild-type strain using quantitative Real-Time-PCR (qRT-PCR). Complementary DNA (cDNA) was obtained from bacteria from each setting by reverse transcription of RNA isolated and purified using the RNeasy Mini kit (Qiagen). Triplicates were performed for each forward and reverse primer pair combination. Primers were purchased from Qiagen (Quantitect Primer Assay) (see Table 2).

Primers were diluted to 1 pmol/µl for further procedure and qRT-PCR was run (7900 HT Fast Real Time System, Applied Biosystems). 16sRNA was used as for normalization and calculation of relative expression.

Threshold cycle values (CT) of the tested genes were determined and normalized expression of each target gene was given as the ΔCT between the log2 transformed CT of the target gene and the log2 transformed CT of the internal control (ACTB). Log2 transformed gene expression levels (ΔCT) of each target gene for intracellular bacteria were expressed as log2 differences from control (= log2 ΔACT method). Data was acquired and analyzed with the SDS 2.3 and RQ-Manager 1.2, respectively.

Animals and insects

Six to eight week-old female BALB/c mice, purchased from Harlan Winkelmann (Borchen, Germany), were kept at our breeding facilities in specific-pathogen-free conditions and used in all experiments. Galleria mellonella larvae, purchased from fauna topics (Marbach, Germany) were reared at 32°C in darkness and on an artificial diet (22% maize germ, 11% dry yeast, 17.5% bee wax, 11% honey and 11% glycerine) prior to use. Last instar larvae, each weighing between 250 and 350 mg, were used in all experiments [20].

Insect and animal models of infection

In all experiments, fresh cultures of bacteria, prepared from an overnight culture, were used. Briefly, bacteria were grown in Brain heart Infusion (BHI) at 37°C, harvested in the exponential growth phase and washed twice with 1 x PBS. The pellet was resuspended in 1 x PBS and the bacterial concentration was calibrated by optical absorption. Further dilutions were prepared in 1 x PBS to obtain required numbers of bacteria for infection. The infection of G. mellonella was performed as previously described by Makkerje et al [20]. Briefly, the human pathogenic L. monocytogenes and its isogenic wp deletion mutants or complemented strains were separately injected (10⁹ CFU/larva) into the hemocoel of the last instar larvae and the infection was monitored at 37°C.

In all experiments, fresh cultures of bacteria, prepared from an overnight culture, were used. Briefly, bacteria were grown in Brain heart Infusion (BHI) at 37°C, harvested in the exponential growth phase and washed twice with 1 x PBS. The pellet was resuspended in 1 x PBS and the bacterial concentration was calibrated by optical absorption. Further dilutions were prepared in 1 x PBS to obtain required numbers of bacteria for infection. Primary mice infections in vivo infection with L. monocytogenes wild-type, Δlmo0515, Δlmo1580, Δlmo2673 or Δlmo0515Al-
mo1580Δlm02673 were performed by an intravenous injection of viable bacteria in a volume of 0.2 ml of 1×PBS. Spleens and livers were harvested three days after infection. Bacterial growth in spleens and livers was determined by plating 10-fold serial dilutions of the organ homogenates on BHI agar plates. Colonies were counted after 24 h of incubation at 37°C.

Ethics statement
This study was carried out in strict accordance with the regulation of the National Protection Animal Act (§7-9a Tierschutzgesetz). The protocol was approved by the local Committee on the Ethics of Animal Experiments (Regierungsbürozirk Mittelhessen) and permission was given by the local authority (Regierungspräsidium Giessen, Permit Number: GI 15/5-Nr.63/2007).

Statistical analysis
All experimental work was performed for a minimum of three times. Significant differences between two values were compared with a paired Student’s t-test. Values were considered significantly different when P<0.05.

Results
Comparative genomic analysis and promoter prediction
All three Usps of L. monocytogenes harbor an UspA domain based on the Pfam analysis (data not shown), and thus represent paralogues of the UspA family of E. coli [1]. The structure and function of UspA is highly conserved among several bacteria and eukaryotic organisms. In line with this, comparative analysis in 19 listerial strains including all serotypes using GECO [25] (cut-off protein identity 80%, 90%) revealed a common chromosomal location for all three usp genes (Figure S1, S2, S3). Previously identified regulatory RNAs [26] were not located in the flanking regions of the usp genes. Furthermore, promoter box analysis of the upstream regions indicated that all usp genes harbour a σB box upstream of their transcriptional start sites (Figure 1).

Universal stress proteins contribute to extracellular survival of L. monocytogenes at low pH condition
L. monocytogenes is able to resist and survive at low pH conditions, which may occur in acidic food, in the gastric milieu and within the macrophage phagosome [27,28]. In order to assess the role of listerial Usps at low pH environment we challenged exponentially grown L. monocytogenes listerial Usps at low pH condition which may occur in acidic food, in the gastric milieu and within the macrophage phagosome [27,28]. In order to assess the role of listerial Usps at low pH condition and the oxidative stress response, the Δlm01580 deletion had the strongest effect on intracellular survival. Compared to the wild-type strain, only 72% of Δlm01580 were able to resist the hostile macrophage environment. Δlm02673 exhibited similar survival attenuation with 76% viable bacteria compared to wild-type strain. No significant effect on intracellular survival was detected in the Δlm02673 mutant (Figure 4). To confirm that the usp genes are intracellular induced we applied quantitative RT-PCR. The analysis showed that usp transcripts obtained from intracellular versus extracellular grown bacteria displayed a strong induction of usp genes (Figure 5).

Decreased virulence of usp mutants in an invertebrate in vivo model
G. mellonella is currently a well established model host for studying the virulence attributes of the human pathogenic L. monocytogenes [20]. In this work, we used this invertebrate model to evaluate the pathogenicity of listerial usp deletion mutants. G. mellonella larvae were infected with pathogenic EGD-e and its isogenic usp mutants namely Δlm01580, Δlm02673 and Δlm0515 respectively. Survival rate of the infected larvae was determined at 37°C for over a period of 7 days.

Attenuated virulence associated with higher survival rates of larvae (Figure 6) were observed in Δlm01580, Δlm02673 and Δlm0515 (75%, 65% and 50% respectively) as compared to wild-type infection (37%). The difference between Δlm0515 and wild-type did however not reach statistical significance. As observed previously with intracellular growth in macrophages, the Δlm01580 mutation had the strongest effect on the pathogenicity, exhibiting nearly avirulent characteristics. A similar result was obtained for the triple mutant Δlm0515Δlm01580Δlm02673 (Figure S4). To confirm that the deletion of usp genes is responsible for the effects in invertebrate infections we complemented the single usp mutants. Complementation restored the ability to kill insect larvae to levels comparable to the wild-type bacteria (Figure S5).

We observed no differences in growth of usp mutants and wild-type strain when cultured in BHI (data not shown) which excludes the possibility that varying numbers of inoculum CFUs of usp deletion mutants affected the survival of insect larvae.

Growth and survival of Listeria usp deletion mutants is profoundly impaired in a murine infection model
Although several studies have investigated the role of Usps in different bacteria, an important role of Usps in vivo has only been
shown for the regulation of growth in *S. typhimurium* [12] and *M. tuberculosis* [6]. To elucidate the influence of listerial Usps on the growth and survival *in vivo*, we injected BALB/c mice with three different usp deletion mutants or wild-type and determined the number of bacteria isolated from the liver. Remarkably, the number of CFU of all mutants compared to wild-type was dramatically diminished in this mouse model (Figure 7). The number of viable bacteria lacking the respective usp gene was significantly lower in livers and spleens of infected mice. In this setting, growth of *Dlmo0515* was reduced to 22% in spleen and 32% in liver as compared to wild-type strain. The number of *Dlmo2673* was reduced to 68% and 60%, and the *Dlmo1580* count declined to 38% and 68%, respectively. The isogenic usp triple mutant showed comparable results as the single usp mutants.

**Discussion**

*L. monocytogenes* is capable of withstanding a variety of stressors encountered in nature and within infected host cells [18]. The ability to rapidly adjust to the changing environment is essential for its pathogenic lifestyle [18]. In this study, we provide strong evidence that universal stress proteins (Usps) are highly conserved among *Listeriae* and are important for resistance and growth of *L. monocytogenes* by investigating usp deletion mutants exposed to different stress conditions both *in vitro* and *in vivo*.

Universal stress proteins were shown to be important in response to stress in gram-negative bacteria, including *E. coli* [31], *S. typhimurium* [12] and *Azospirillium brasilense* [32]. However, the role of these proteins in gram-positive bacteria is not as well delineated. We have previously revealed an induction of three genes, *lmo0515*, *lmo2673* and *lmo1580* in *L. monocytogenes* in response to heat shock and acid stress [33,34]. In accordance with effects described for *E. coli* lacking the *uspA* gene, *L. monocytogenes* usp deletion mutations investigated in this study displayed impaired capability to resist exposure to *H₂O₂* and low pH conditions. Variable susceptibility to stress conditions between deletion mutants may represent a non-redundant role of Usps in stress responses. Similar observations were made for *E. coli* lacking the *uspA* gene, *L. monocytogenes* usp deletion mutations investigated in this study displayed impaired capability to resist exposure to *H₂O₂* and low pH conditions. Variable susceptibility to stress conditions between deletion mutants may represent a non-redundant role of Usps in stress responses. Similar observations were made for *E. coli* and addressed in a recent study testing several usp deletion mutants [31]. Nachin et al. uncovered that the UspA proteins differ in their responses to oxidative stress and DNA damaging agents and defined similarities as well as differences in expression pattern based on their biological role in stress response [31]. Thus, certain environmental changes may not require the induction of all usp genes, but allow adjustment depending on the specialized role of a particular Usp.

Generally, usp genes are expressed in monocistronic units and the production of UspA seems to be primarily regulated at the

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**Figure 1. Transcriptional overview of chromosomal loci of *lmo0515* (A), *lmo1580* (B), and *lmo2673* (C) derived from sequencing data of *L. monocytogenes* (data not shown).** σB boxes are depicted as orange arrows, the primary transcripts from their start sites by grey bars, the ribosome binding site as a yellow box and the respective coding sequence from their start sites by green bars. doi:10.1371/journal.pone.0024965.g001
level of transcription initiation [1]. We and others have previously reported an important role for σB, a transcription factor that controls an inducible regulon in L. monocytogenes induced following challenge to different stress conditions, such as acids, high osmolarity or carbohydrate starvation [17,34,35]. As previously reported, a single σB promoter has been identified for lmo1580; and promoter analysis revealed σB boxes for lmo2673 and lmo0515. Thus, it appears likely that σB possesses a major role in stress induced transcriptional regulation of usp genes in L. monocytogenes. Furthermore, phenolic acids and fructose-6-phosphate were shown to influence usp gene expression in Lactobacillus plantarum and E coli, respectively. L. monocytogenes mobilizes a large set of metabolic genes in response to stress [24]. Especially, genes of the carbohydrate metabolism are strongly expressed. Possible effects

Figure 2. Effect of low acidic condition (pH = 2.5) on survival of usp deletion mutants compared to L. monocytogenes EGD-e wild-type. ∆lmo0515 and ∆lmo1580 display decreased growth at all observations, while impaired resistance in ∆lmo2673 was visible only at 20 min post challenge. Statistically significant differences were identified using a two-tailed Student t test. (*, p<0.05 and **, p<0.005).

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Figure 3. Survival of L. monocytogenes EGD-e wild-type and usp deletion mutants after exposure to H2O2. As appreciable, resistance of all deletion mutants was strongly impaired, resulting in decreased growth as compared to wild-type strain. Statistically significant differences were identified using a two-tailed Student t test. (*, p<0.05; **, p<0.005, and *** p<0.0005).

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of metabolic constituents on usp gene regulation may therefore not be excluded and require further investigation. [15,36].

In this regard, the temporal and coordinated activities of different Usps of L. monocytogenes may enable the bacteria to survive in acid-rich niches such as in the upper intestinal tract or mediate resistance to oxidative stress and bile acids in the spleen and liver.

Following ingestion by phagocytic cells, Listeria are faced with the hostile environment in the phagosome [18]. As shown previously, virulence and intracellular growth adaption of L. monocytogenes depends on a concerted gene expression program including the up-regulation of universal stress proteins [24,37]. In this study, we demonstrate that survival of Listeriae in murine macrophages is dependent on the presence of Usps. This was reflected by decreased virulence, resulting in a significant reduction of bacteria lacking the respective usp genes. In addition we observed intracellular transcriptional induction of all three usp genes which supports the

![Figure 4](image-url) **Figure 4.** The intracellular survival of L. monocytogenes EGD-e wild-type and usp deletion mutants was assessed in murine macrophages according to [30]. Intracellular bacteria burden was decreased in macrophages inoculated with Δmo1580 and Δmo2673, but no effect was visible for Δmo0515. Statistically significant differences were identified using a two-tailed Student t test. (*, p<0.05 and **, p<0.005). doi:10.1371/journal.pone.0024965.g004

![Figure 5](image-url) **Figure 5.** qRT-PCR analysis of lmo0515, lmo1580 and lmo2673 expressed in L. monocytogenes EGD-e. The graph displays the relative expression of the respective usp gene in the intracellular vs. extracellular setting. High fold changes indicate that the particular gene was higher expressed in intracellularly localized bacteria as compared to expression in bacteria that were grown in BHI. The expression of lmo0515 and lmo2673 is strongly enhanced in intracellular pathogens, while levels of lmo1580 remain relatively constant. This is consistent with the observation made in this study, which shows a strong dependence on lmo2673 and lmo0515 in the murine infection model experiments as compared to extracellular in vitro challenges, while lmo1580 seems to play an important role in both conditions. doi:10.1371/journal.pone.0024965.g005
evidence that the particular stress proteins are involved in the intracellular pathogenic lifestyle of *L. monocytogenes*.

The impact of Usps in infection has only been examined for a few bacteria and their role, if any, in gram-positive pathogens has not been described [6,12,31,37]. For this reason, we further characterized the effect of listerial Usps in *in vivo* infection models. First we tested their effects on the viability of *G. mellonella* larvae, a recently described alternative infection model for *L. monocytogenes*.

**Figure 6. Survival of *G. mellonella* larvae after inoculation with usp deletion mutants.** As compared to *L. monocytogenes* EGD-e wild-type, all usp deletion mutants exhibited impaired virulence, resulting in higher survival rates of *G. mellonella* larvae. Strongest effects were observed for Δlmo1580 and Δlmo2673, while deletion of lmo0515 was not associated with significant increase in survival of *G. mellonella*. Results represent mean values of at least three independent experiments with a total of 80 larvae per treatment. Statistically significant differences were identified using a two-tailed Student t test. (***, p<0.0005 and ns-not significant).

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**Figure 7. Survival rates of intravenously inoculated *L. monocytogenes* EGD-e or usp deletion mutants in mice livers and spleens.** Survival of mutant bacteria are impaired in both organs, reflecting a role of Usps in listerial resistance and growth *in vivo*. Statistically significant differences were identified using a two-tailed Student t test. (*, p<0.05).

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Figure S2 Comparative analysis of flanking regions of listerial universal stress protein lmo1580 among 19 L. monocytogenes species including all known serotypes.

(PDF)

Figure S3 Comparative analysis of flanking regions of listerial universal stress protein lmo2673 among 19 L. monocytogenes species including all known serotypes.

(PDF)

Figure S4 Survival of G. mellonella larvae after inoculation with usp deletion mutants. As compared to L. monocytogenes EGD-e wild-type, triple deletion mutant of lmo1580, lmo2673 and lmo0515 exhibited impaired virulence similar to the single gene deletion mutant lmo1580, resulting in significant higher survival rates of G. mellonella larvae. Results represent mean values of at least three independent experiments and each repetition contained 30 larvae per treatment. Statistically significant differences were identified using a two-tailed Student t test. (***, p<0.005).

(TIF)

Figure S5 Complementation of lmo1580, lmo2673 and lmo0515 into their respective isogenic deletion mutants lmo1580, lmo2673 and lmo0515 resulted in induced virulence. Artificial introduction of the individual usp genes lmo1580, lmo2673 and lmo0515 into their respective isogenic deletion mutants lmo1580, lmo2673 and lmo0515 resulted in an increase of virulence similar to the wild-type EGD-e. Results represent means of at least three independent determinations ± standard deviations. Each repetition contained 20 larvae per treatment.

(TIF)

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Author Contributions

Conceived and designed the experiments: CSG BI FP WM MAM KM YT TC TH. Performed the experiments: CSG BI FP WM MAM. Analyzed the data: CSG BI MAM KM YT TC TH. Wrote the paper: BI FP TH TC. IT support and contributed to the manuscript: AB.

Supporting Information

Figure S1 Comparative analysis of flanking regions of listerial universal stress protein lmo0515 among 19 L. monocytogenes species including all known serotypes.

(PDF)

References
