The cell envelope stress response mediated by the LiaFSR$_{Lm}$ three-component system of *Listeria monocytogenes* is controlled via the phosphatase activity of the bifunctional histidine kinase LiaS$_{Lm}$

Frederike Fritsch, Norman Mauder,† Tatjana Williams,† Julia Weiser, Markus Oberle and Dagmar Beier

Theodor-Boveri-Institut für Biowissenschaften, Lehrstuhl für Mikrobiologie, Universität Würzburg, Am Hubland, D-97074 Würzburg, Germany

Most members of the phylum Firmicutes harbour a two-component system (TCS), LiaSR, which is involved in the response to cell envelope stress elicited most notably by inhibitors of the lipid II cycle. In all LiaSR systems studied in detail, LiaSR-mediated signal transduction has been shown to be negatively controlled by a membrane protein, LiaF, encoded upstream of *liaSR*. In this study we have analysed the LiaSR orthologue of *Listeria monocytogenes* (LiaSR$_{Lm}$). Whole-genome transcriptional profiling indicated that activation of LiaSR$_{Lm}$ results in a remodelling of the cell envelope via the massive upregulation of membrane-associated and extracytoplasmic proteins in the presence of inducing stimuli. As shown for other LiaSR TCSs, LiaSR$_{Lm}$ is activated by cell wall-active antibiotics. We demonstrate that the level of phosphorylated LiaR$_{Lm}$, which is required for the induction of the LiaSR$_{Lm}$ regulon, is controlled by the interplay between the histidine kinase and phosphatase activities of the bifunctional sensor protein LiaS$_{Lm}$. Our data suggest that the phosphatase activity of LiaS$_{Lm}$ is stimulated by LiaF$_{Lm}$ in the absence of cell envelope stress.

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**INTRODUCTION**

*Listeria monocytogenes* is a Gram-positive saprophytic bacterium which can be isolated from many environmental sources, such as soil, water, a variety of foodstuffs, and human and animal faeces. Due to its facultative intracellular lifestyle, *L. monocytogenes* can cause serious infections in humans and other vertebrates. Pregnant women and immunocompromised individuals are at particular risk of experiencing fatal listeriosis, characterized by abortion or stillbirth, life-threatening sepsicaemia and meningocoeephyalitis (Vázquez-Boland et al., 2001). Consistent with its widespread occurrence in the environment and the need to cope with changing environmental conditions, *L. monocytogenes* harbours a vast number of transcriptional regulators, including 14 two-component systems (TCSs) and an orphan response regulator (RR) (Glaser et al., 2001). Signal transduction by a TCS initiates with the autophosphorylation of a conserved histidine residue in the transmitter domain of the sensor histidine kinase (HK), which is triggered by a specific input signal usually perceived by an N-terminal sensor domain. The phosphoryl group is then transferred to an aspartic acid residue in the receiver domain of the cognate RR. Phosphorylation of the RR controls its propensity to act as a transcriptional activator or repressor of target genes. Signal transduction is shut off by spontaneous hydrolysis of the phosphorylated RR (RR–P) or by dephosphorylation via the phosphatase activity towards the RR–P of either the cognate HK or another specific phosphatase (Gao & Stock, 2009).

To date, the *Listeria* TCSs AgrAC, VirRS, ResDE, LisRK, CesRK and the orphan RR DegU have been analysed. DegU (Lmo2515) is involved in the temperature-responsive regulation of motility and chemotaxis genes by controlling the expression of the anti-repressor protein GmaR (Kamp & Higgins, 2009; Williams et al., 2005b; Gueriri et al., 2008a, b; Mauder et al., 2008; Shen et al., 2006). The quorum-sensing TCS AgrAC (Lmo0051–Lmo0050) controls
adhesion and the early steps of biofilm formation (Rieu et al., 2007, 2008; Riedel et al., 2009). The RR ResD (Lmo1948) is involved in the control of respiration and carbon source utilization (Larsen et al., 2006), while the RR VirR (Lmo1745) contributes to virulence by regulating the modification of cell surface components (Mandin et al., 2005). The TCSs CesRK (Lmo2422–Lmo2421) and LisRK (Lmo1377–Lmo1378) are involved in the cell envelope stress response of L. monocytogenes. Deletion mutants of cesR and cesK exhibit an increased resistance towards ethanol as well as increased sensitivity towards antibiotics of the β-lactam family (Kallipolitis et al., 2003). Deletion of lisK results in increased ethanol tolerance, increased acid resistance in stationary phase, a reduced capability to tolerate environments of elevated osmolarity, enhanced resistance to the lantibiotic nisin and a greatly enhanced sensitivity to cephalosporins (Sleator & Hill, 2005; Cotter et al., 1999, 2002).

L. monocytogenes contains another TCS, Lmo1021–Lmo1022, which is thought to respond to perturbations of cell envelope architecture. Lmo1021–Lmo1022 is orthologous to the LiaSR TCS of Bacillus subtilis, which is induced by cell wall-active antibiotics that interfere with the lipid II cycle in the cytoplasmic membrane. Other inducing stimuli perceived by LiaSR are alkaline shock and the presence of cationic antimicrobial peptides, organic solvents, ethanol and detergents (Mascher et al., 2004; Mascher, 2006). The HK LiaS belongs to the class of intramembrane-sensing histidine kinases (IM-HKs) mainly found in Gram-positive bacteria, which are characterized by an N-terminal sensing domain consisting of two deduced transmembrane helices with a spacing of less than 25 amino acids and the absence of signalling modules in the cytoplasmic part of the HK (Mascher, 2006). It has been shown that LiaR positively regulates transcription of the liaHGFSR locus, with liaI and liaH encoding a small, presumably membrane-bound protein and a homologue of the Escherichia coli phage shock protein A, respectively, the yheYZ–yhdA operon encoding a TCS and an oxidoreductase, and ydhE encoding a putative glucosyltransferase (Jordan et al., 2006; Wolf et al., 2010). Interestingly, the kinase activity of LiaS seems to be controlled via an inhibitory interaction with the membrane protein LiaF, which is encoded upstream of liaSR. This inhibitory interaction is relieved in the presence of cell envelope stress (Jordan et al., 2006). Orthologues of LiaF are present in the genomes of all species that harbour orthologues of LiaSR (Mascher, 2006). Orthologues of the LiaFSR system have already been characterized in Staphylococcus aureus, Streptococcus mutans, Streptococcus pneumoniae and Lactococcus lactis (Suntharalingam et al., 2009; Martínez et al., 2007; Kuroda et al., 2003; Belcheva & Golemi-Kotra, 2008; Eldholm et al., 2010).

In this study we will refer to the Lmo1021–Lmo1022 TCS of L. monocytogenes as LiaSR_Lmo. We report the characterization of L. monocytogenes of the LiaSrLmo regulon, which consists mainly of genes encoding membrane-associated or putative secreted proteins. We demonstrate that, as reported for other LiaSR orthologues, transcription of LiaSrLmo target genes is induced by the exposure of L. monocytogenes to cell wall-active antibiotics. Furthermore, our results suggest that the LiaF orthologue Lmo1020 controls signal transduction in the Listeria system by affecting the phosphatase activity of the HK LiaS_Lmo.

**METHODS**

**Bacterial strains and growth conditions.** L. monocytogenes sv1/2a EGD was obtained from S. H. E. Kaufmann (Max-Planck-Institut für Infektionsbiologie, Berlin, Germany). L. monocytogenes ΔliaR carrying an in-frame deletion of the RR gene lmo1022 has been described previously (Williams et al., 2005a), and will be referred to as L.m. ΔliaR in this study. L. monocytogenes was grown at 37°C in brain heart infusion (BHI) broth or on BHI agar plates. When required, erythromycin was added to a final concentration of 10 μg ml⁻¹. E. coli strains DH5α, BL21 and M15 (pREP4) were grown in Luria–Bertani (LB) broth. When necessary, antibiotics were added to the following final concentrations: ampicillin, 100 μg ml⁻¹; kanamycin, 50 μg ml⁻¹; erythromycin, 300 μg ml⁻¹.

**Construction of L. monocytogenes mutants L.m. ΔliaS, L.m. ΔiaaS, L.m. ΔiaaR, L.m. ΔiaaR, and L.m. ΔiaaS-D54Y.** All Listeria mutants were constructed using a two-step integration/excision procedure which is based on transformation of Listeria with an appropriate mutagenesis plasmid derived from pLSV1 (Wuenscher et al., 1991), as described previously (Williams et al., 2005a). If not stated otherwise, L. monocytogenes sv1/2a EGD was used as the parent strain. All PCR fragments used for the construction of mutagenesis plasmids were subjected to automated sequencing to ensure proper PCR amplification. The oligonucleotides used as primers in the PCRs are listed in Supplementary Table S1. Mutant L.m. ΔliaS has an in-frame deletion of the region encoding amino acids 3–276 of the HK LiaS_Lmo. To construct the knockout plasmid pLSV-1021, a 524 bp SalI–NcoI fragment PCR-amplified with primer pair 1021-1/1021-2 and derived from ORF lmo1020, and a 437 bp Ncol–BamHI fragment PCR-amplified with primer pair 1021-3/1021-4 and comprising the region encoding amino acids 277–352 of LiaS_Lmo and amino acids 1–68 of LiaS_Lmo, were cloned into pLSV1 vector DNA. The complemented strain L.m. liaS_D54Y was obtained by transformation of L.m. ΔliaS with plasmid pLSV-1021c carrying a 1777 bp SalI–BamHI fragment PCR-amplified with primer pair 1021-1/1021-4 and comprising the liaS_Lmo gene as well as 524 and 200 bp from its 5’ and 3’ flanking sequences, respectively. Mutant L.m. liaS_D54Y expresses a derivative of RR LiaR_Lmo with a substitution of the aspartic acid residue at position 54 by tyrosine. The mutated liaR allele was obtained by recombinant PCR (Ho et al., 1989) using oligonucleotides 1022c-1 and 1022c-2 as outer primers together with appropriate primers to introduce the desired point mutation. After subcloning into pSL1180 (Brosius, 1989) and sequencing, the 1482 bp BamHI fragment encoding LiaR_D54Y was ligated into pLSV1, yielding mutagenesis plasmid pLSV-1022_D54Y, which was used to transform mutant L.m. ΔliaR (Williams et al., 2005a). Mutant L.m. ΔliaS,liaaS_D54Y was obtained by transformation of L.m. liaaS_D54Y with suicide plasmid pLSV-1021. Knockout plasmid pLSV-1020 contained a 565 bp EcoRI–Xhol fragment derived from the upstream region of lmo1020 (liaF), including the start codon (amplified with primer pair lmo1020-1/lmo1020-2) and a 652 bp Xhol–BamHI fragment comprising the last codon of lmo1020 and codons 1–220 of LiaS_Lmo (amplified with primer pair lmo1020-3/lmo1020-4). pLSV-1020 was used to transform strain L.m. ΔliaaR to yield the double mutant L.m. ΔliaaS,ΔliaaR.
Knockout plasmids pLSV-pta and pLSV-ackA were used to construct deletions of pta and ackA in strain L.m. A liaS vector was used to overexpress LiaS in a two-step mutagenesis procedure and were created as follows: EcoRI–XhoI and XhoI–BamHI DNA fragments of 650 and 808 bp derived from the immediate upstream and downstream regions of pta, respectively, were PCR-amplified with primer pairs pta-1/pta-2 and pta-3/pta-4 and were cloned into pLSV1, yielding plasmid pLSV-pta. To obtain pLSV-ackA, a 656 bp EcoRI–XhoI fragment corresponding to the upstream region of ackA and a 666 bp XhoI–BamHI fragment comprising 53 bp of the 3′ coding region and 605 bp of the downstream region of ackA were cloned into pLSV1. These DNA fragments were PCR-amplified with primer pairs ackA-1/ackA-2 and ackA-3/ackA-4, respectively.

Transformation of L. monocytogenes and temperature shift to 42 °C yielded erythromycin-resistant clones harbouring a chromosomal integration of the respective mutagenesis plasmid. Erythromycin-sensitive clones obtained after prolonged subculturing at 30 °C were screened by PCR for the second recombination event resulting in the desired mutation, and were further checked by sequence analysis of appropriate PCR fragments.

**Construction of plasmids expressing recombinant LiaR<sub>Lm</sub> and LiaS<sub>Lm</sub> and purification of fusion proteins.** A 632 bp DNA fragment encoding the transmitter domain (amino acids 146–352) of the HK LiaS<sub>Lm</sub> was amplified with primer pair 1021-E5/1021-E3, generating BamHI sites at the 5′ and 3′ termini. The DNA fragment was ligated into BamHI-digested pGEX–3X vector DNA. A plastid clone containing the BamHI fragment in the desired orientation, generating an in-frame fusion of the vector-borne glutathione S-transferase (GST) gene (gst) and the ORF encoding the transmitter domain of LiaS<sub>Lm</sub> was selected by restriction of plastid DNA with EcoRI and was designated pGEX-1021. To generate plastid pQE-1022, a 666 bp DNA fragment encoding the RR LiaR<sub>Lm</sub> was PCR-amplified with primer pair lmo1022-5/lmo1022-3, generating BamHI and PstI restriction sites at the 5′ and 3′ ends of the fragment. The DNA fragment was ligated into pQE30 vector DNA, creating an N-terminal His<sub>6</sub> tag. The fusion protein GST–LiaS<sub>Lm</sub> was produced in E. coli BL21 and purified by affinity chromatography on glutathione-Sepharose 4B, as described previously (Beier & Frank, 2000). His<sub>6</sub>–LiaR<sub>Lm</sub> was overproduced in E. coli M15 (pREP4) (Qiagen), and was purified by affinity chromatography on glutathione–agarose columns (Qiagen) was carried out following the manufacturer’s recommendations. The recombinant His<sub>6</sub>–LiaS<sub>Lm</sub> protein was stored in 50 mM Tris/HCl, pH 7.5, 0.2 M NaCl, 50 mM MgCl<sub>2</sub>, 10 mM CaCl<sub>2</sub>, 30% (v/v) glycerol.

**In vitro phosphorylation assays.** In vitro phosphorylation assays using multiple turnover conditions were performed in a final volume of 25 μl reaction buffer [50 mM Tris/HCl, pH 7.5, 50 mM KCl, 10 mM MgCl<sub>2</sub>, 0.2 M ATP, 250 mM [γ-<sup>35</sup>P]ATP (3000 Ci mmol<sup>−1</sup>; 111 TBq mmol<sup>−1</sup]) containing the HK and RR at 1 and 6 μM, respectively. The reaction mixtures were incubated at 37 °C for 15 min. Phosphotransfer reactions from GST–LiaS<sub>Lm</sub>–P to His<sub>6</sub>–LiaR<sub>Lm</sub> were performed on ice and were initiated by the addition of the RR protein to GST–LiaS<sub>Lm</sub> that had been incubated for 15 min at 37 °C in reaction buffer containing 250 mM [γ-<sup>35</sup>P]ATP. The reactions were stopped by the addition of sample buffer [60 mM Tris/HCl, pH 7.5, 50 mM Na<sub>2</sub>EDTA, 10% (v/v) glycerol, 2% SDS, 5% β-mercaptoethanol, 0.05% bromophenol blue], and the reaction mixtures were separated by electrophoresis on a 12% SDS-polyacrylamide gel (1.5 mm).

**Preparation of RNA and quantitative real-time PCR (qRT-PCR).** cDNA was isolated from L. monocytogenes as described previously (Williams et al., 2005b). cDNA was prepared from 5 μg RNA using SuperScript II reverse transcriptase (Invitrogen). cDNA was then amplified using Q-PCR Mastermix SYBR Green (Thermo Fisher Scientific) on a StepOne Plus Real-Time system (AB Biosystems). The relative expression levels of the genes studied were normalized to the housekeeping gene rpoB. Data were analysed using the ΔΔC<sub>T</sub> method.

Two qRT-PCR experiments were performed in duplicate with cDNA which was reverse-transcribed from two independent RNA preparations.

**Microarray hybridization and data analysis.** RNA for microarray experiments was isolated from L. monocytogenes cultures grown to OD<sub>600</sub> 1.0. cDNA synthesis and hybridization of custom-made whole-genome microarrays containing 70mer oligodeoxynucleotides covering all ORFs of the L. monocytogenes EGD-e genome were performed essentially as described previously (Williams et al., 2005b). Raw TIF images were scanned with an Axon GenePix 4200 Microarray scanner (Molecular Devices). Spot grids were manually fitted to the microarray images. Microarrays were analysed using Limma (Smyth & Speed, 2003) implemented in the R language.

**Mapping of transcriptional start sites and electrophoretic mobility shift assays (EMSAs).** Transcriptional start sites were mapped by rapid amplification of 5′ cDNA ends (5′-RACE) using the 5′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen) according to the manufacturer’s recommendations. The oligonucleotide primers used, which are specific for lmo0047, lmo0193, lmo0954, lmo1966, lmo2258 and lmo2568, are listed in Supplementary Table S1. Single-stranded oligonucleotides 2568-a and 2568-b (Supplementary Table S1) were labelled using the Biotin 3′ End DNA labelling kit (Pierce) and were subsequently annealed to form the DNA probe used for EMSA experiments. EMSA was performed using the LightShift Chemiluminescent EMSA kit (Pierce) according to the manufacturer’s instructions. In vitro phosphorylation of His<sub>6</sub>–LiaR<sub>Lm</sub> was performed as described previously (Dietz et al., 2002).

**RESULTS**

**RR LiaR<sub>Lm</sub> is phosphorylated by the HK LiaS<sub>Lm</sub> in vitro**

The genes liaSR<sub>Lm</sub> belong to the transcriptional unit lmo1020–lmo1023, which also comprises a liaIF orthologue (lmo1020) and a gene encoding a protein of unknown function (lmo1023). To investigate the interaction between the HK LiaS<sub>Lm</sub> and the RR LiaR<sub>Lm</sub> in vitro phosphorylation assays with the purified recombinant proteins were performed. LiaS<sub>Lm</sub> is predicted to be anchored to the cytoplasmic membrane via two transmembrane domains flanking a short extracytoplasmic loop (DAS – Transmembrane Prediction Server; http://www.sbc.su.se/~miklos/DAS/tmdas.cgi). The part of its transmembrane domain comprising amino acids 146–352 was fused to an N-terminal GST tag. The cognate RR LiaR<sub>Lm</sub> was expressed with an N-terminal His<sub>6</sub> tag. Incubation of GST–LiaS<sub>Lm</sub> in the presence of [γ-<sup>35</sup>P]ATP resulted in the autophosphorylation of the HK (Fig. 1), while His<sub>6</sub>–LiaR<sub>Lm</sub> was not phosphorylated under these conditions (data not shown). When GST–LiaS<sub>Lm</sub> and His<sub>6</sub>–LiaR<sub>Lm</sub> were combined under multiple turnover conditions, complete dephosphorylation of GST–LiaS<sub>Lm</sub>–P was observed due to the phosphoryl group transfer to His<sub>6</sub>–LiaR<sub>Lm</sub>, however, the phosphorylated RR protein did not accumulate to significant levels (Fig. 1a). Phosphorylated His<sub>6</sub>–LiaR<sub>Lm</sub> could only be detected within the first 90 s of incubation on ice upon its treatment with the phosphoryl group transfer factor.
addition to GST–LiaS<sub>Lm</sub>, that had been pre-treated with [γ-<sup>32</sup>P]ATP for 15 min (Fig. 1b), indicating rapid hydrolysis of LiaR<sub>Lm</sub>~P in the presence of LiaS<sub>Lm</sub>.

### Whole-genome profiling of mutants L.m. ΔliaS and L.m. ΔliaR

To characterize the regulon controlled by the TCS LiaSR<sub>Lm</sub>, the mutant L.m. ΔliaS carrying an in-frame deletion of the HK gene liaS<sub>Lm</sub> was constructed. Whole-genome microarray analysis was performed with the mutant using a custom-made whole-genome microarray containing 70mer oligodeoxynucleotides that covered all ORFs of the L. monocytogenes EGD-e genome (Williams et al., 2005b). Cy5- and Cy3-labelled cDNA was prepared from five independent RNA preparations of mutant L.m. ΔliaS and wild-type L. monocytogenes, respectively, and was hybridized to seven microarray slides, creating 28 sets of hybridization data. Applying a signal ratio cut-off of <0.5 and >2.0, 29 genes belonging to 16 different transcriptional units were found to be upregulated in the HK mutant, including the lmo1020–lmo1023 operon. Although cotranscription of ORFs lmo2567 and lmo2568 was not reported in the study of Toledo-Arana et al. (2009), the very similar relative expression ratios observed in our microarray experiment, and the fact that the intergenic region spans only 22 bp and transcription terminators are predicted upstream of lmo2568 and downstream of lmo2567, suggest that these genes belong to an operon. The differentially transcribed genes encode mainly proteins of unknown function and putative components of ABC transporters (Table 1). The gene products of 18 genes are predicted to be associated with the cytoplasmic membrane, and another three gene products are non-cytoplasmic proteins due to the presence of a signal peptide (PSORTb, ExPaSy Proteomics Server). Target genes lmo0954 and lmo0955 are orthologues of liaIH, which form an operon with liaG, liaF and the TCS liaSR in B. subtilis. No downregulated genes were detected in the microarray analysis of L.m. ΔliaS. Transcription of 16 target genes representing different transcriptional units was analysed by qRT-PCR, and with the exception of lmo0181 and lmo2015, their differential expression in L.m. ΔliaS was confirmed (Table 1). Unexpectedly, transcriptome analysis of the RR deletion mutant L.m. ΔliaR, which was performed using cDNA from two independent RNA preparations and four microarray slides (creating 16 sets of hybridization data), revealed that a subset of 20 of the genes differentially expressed in L.m. ΔliaS was downregulated as compared with the wild-type L. monocytogenes EGD. These data suggested that, despite the derepression of target genes in the mutant lacking the cognate HK LiaS<sub>Lm</sub>, LiaR<sub>Lm</sub> acts as a transcriptional activator of the LiaSR<sub>Lm</sub> regulon. To confirm that differential gene expression in L.m. ΔliaS was due to the deletion of the HK gene, liaS was reintroduced by allelic replacement into mutant L.m. ΔliaS to create the complemented strain L.m. liaS<sup>+</sup>. The transcript amounts of selected target genes (lmo0047, lmo0953 and lmo2568) were compared in L.m. liaS<sup>+</sup> and the wild-type strain via qRT-PCR, and proved to be similar (data not shown).

## Transcription of LiaSR<sub>Lm</sub> target genes is induced in the presence of cell wall-active antibiotics

Since the LiaSR TCS of B. subtilis is activated by cell wall-active antibiotics (Mascher et al., 2004), we investigated the response of the LiaSR<sub>Lm</sub> regulon to several antibiotics.
which target the cell envelope. *L. monocytogenes* cells were grown to OD<sub>600</sub> 0.6, and then vancomycin, bacitracin, fosfomycin and polymyxin were added at concentrations which had been previously shown to either affect or not affect bacterial growth in liquid culture. Incubation of the cultures was then continued for 1 h. Bacteria from untreated control cultures were either harvested at OD<sub>600</sub> 0.6 or after an additional 1 h of growth, depending on whether the effect of inhibitory or subinhibitory concentrations of antibiotics was to be analysed. RNA was prepared and the transcript amounts of the target genes Lmo0955, Lmo1966, Lmo2486 and Lmo2568 were determined by qRT-PCR in comparison with *L. monocytogenes* from untreated culture. Growth in the presence of inhibitory concentrations of vancomycin, fosfomycin and bacitracin (30 μg ml<sup>-1</sup>, 300 μg ml<sup>-1</sup> and 700 μg ml<sup>-1</sup>, respectively) resulted in a pronounced induction of transcription of the tested target genes (Fig. 2), with

### Table 1. ORFs of the LiaSR<sub>Lm</sub> regulon

<table>
<thead>
<tr>
<th>ORF</th>
<th>Function</th>
<th>Genome organization</th>
<th>Microarray ratio, LiaS/wild-type</th>
<th>qRT-PCR ratio, LiaS/wild-type</th>
<th>Microarray ratio, LiaR/wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td>lmo0047</td>
<td>Unknown</td>
<td>Monocistronic</td>
<td>5.00</td>
<td>15.0</td>
<td>0.35</td>
</tr>
<tr>
<td>lmo0181</td>
<td>Unknown, similar to sugar ABC transporter</td>
<td>op 29 (lmo0181–0184)</td>
<td>3.33</td>
<td>11.4</td>
<td>0.43</td>
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<td>lmo0193</td>
<td>Unknown</td>
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<td>3.39</td>
<td>0.41</td>
<td>0.39</td>
</tr>
<tr>
<td>lmo0194</td>
<td>ABC transporter, ATP-binding protein</td>
<td>op 29 (lmo0194–0195)</td>
<td>3.07</td>
<td>0.39</td>
<td>0.29</td>
</tr>
<tr>
<td>lmo0195</td>
<td>Unknown, similar to membrane protein</td>
<td>op 149 (lmo0195–0196)</td>
<td>7.10</td>
<td>0.29</td>
<td>0.26</td>
</tr>
<tr>
<td>lmo0955</td>
<td>liaI</td>
<td>op 149 (lmo0955–0956)</td>
<td>6.78</td>
<td>0.45</td>
<td>0.26</td>
</tr>
<tr>
<td>lmo1020</td>
<td>liaF</td>
<td>op 163 (lmo1018–1023)</td>
<td>2.95</td>
<td>6.8</td>
<td>0.45</td>
</tr>
<tr>
<td>lmo1021</td>
<td>liaS, two-component sensor HK</td>
<td>op 163 (lmo1021–1022)</td>
<td>2.23</td>
<td>0.16</td>
<td>0.26</td>
</tr>
<tr>
<td>lmo1022</td>
<td>liaR, two-component RR</td>
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<td>2.13</td>
<td>0.16</td>
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<tr>
<td>lmo1023</td>
<td>Unknown, similar to K&lt;sup&gt;+&lt;/sup&gt;-uptake system</td>
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<td></td>
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<td>lmo1636</td>
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<td>3.60</td>
<td>12.9</td>
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<td>lmo1657</td>
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<td>3.47</td>
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<tr>
<td>lmo1746</td>
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<tr>
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<td>lmo1966</td>
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<td>lmo2015</td>
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<td>lmo2207</td>
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<tr>
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<td>24.4</td>
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<tr>
<td>lmo2485</td>
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<td>0.26</td>
</tr>
<tr>
<td>lmo2486</td>
<td>Unknown</td>
<td>op 2486 (lmo2486–2487)</td>
<td>7.39</td>
<td>41.0</td>
<td>0.21</td>
</tr>
<tr>
<td>lmo2487</td>
<td>Unknown</td>
<td>op 2487 (lmo2487–2488)</td>
<td>8.50</td>
<td>0.23</td>
<td>0.26</td>
</tr>
<tr>
<td>lmo2567</td>
<td>Unknown</td>
<td>op 2567 (lmo2567–2568)</td>
<td>9.41</td>
<td>0.27</td>
<td>0.26</td>
</tr>
<tr>
<td>lmo2568</td>
<td>Unknown</td>
<td>op 2568 (lmo2568–2569)</td>
<td>11.69</td>
<td>296.5</td>
<td>0.25</td>
</tr>
<tr>
<td>lmo2745</td>
<td>Unknown, similar to ABC transporter (ATP-binding protein)</td>
<td>op 2745 (lmo2745–2746)</td>
<td>2.41</td>
<td>6.3</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Genes whose transcription according to microarray analysis differed more than twofold (ratio >2.0 or <0.5) in the *L. monocytogenes* HK mutant *Lm.* LiaS as compared with the wild-type strain EGD are listed. ORF numbers and functional annotation are according to the ListiList database (http://genolist.pasteur.fr/ListiList/). Prediction of transcriptional units is according to Toledo-Arana et al. (2009). ORFs whose gene products are predicted to be membrane or extracytoplasmic proteins according to PSORTb (ExPASy Proteomics Server) are listed in bold type. The differential transcription of selected ORFs representing the different transcriptional units was analysed by qRT-PCR and, with the exception of lmo0181 and lmo2015, could be verified. The relative expression ratios obtained by qRT-PCR are given in column 5. The primer pairs generating the respective amplicons are listed in Supplementary Table S1. Column 6 lists the relative expression ratios of those genes which were differentially transcribed according to microarray analysis in the RR mutant *L.m.* LiaR as compared with the wild-type strain EGD.
bacitracin eliciting the strongest response. Bacitracin at a subinhibitory concentration (100 μg ml⁻¹) triggered a three- and 10-fold increase in transcription of lmo2486 and lmo2568, respectively, while transcription of lmo0955 and lmo1966 was not significantly altered. Similarly, in the presence of a subinhibitory concentration of vancomycin (0.5 μg ml⁻¹), only a slight increase in the transcript amount of lmo2568 was observed, while transcription of lmo0955, lmo1966 and lmo2486 remained unaffected. However, the presence of fosfomycin in a subinhibitory concentration (20 μg ml⁻¹) resulted in a transcriptional induction similar to that observed when this antibiotic was added at an inhibitory concentration (data not shown). Polymyxin at an inhibitory concentration (500 μg ml⁻¹) also activated the LiaSRₐₐ TCS; however, the transcriptional induction triggered by the presence of this antibiotic was less pronounced (Fig. 2). Subinhibitory concentrations of polymyxin did not affect the transcription of the analysed LiaSRₐₐ target genes (data not shown).

Comparison of the transcript amounts of selected target genes in the mutant L.ₐₐ DiaR grown in the absence and presence of vancomycin demonstrated that transcriptional induction of the LiaSRₐₐ regulon is strictly dependent on the presence of the RR LiaRₐₐ, since no increase in transcription was observed in L.ₐₐ DiaR upon treatment with vancomycin (Fig. 3a). Furthermore, compared with the wild-type, significantly lower amounts of transcript were detected in L.ₐₐ DiaR in the presence of vancomycin, fosfomycin and polymyxin (Fig. 3b). In order to test

![Fig. 2. Expression analysis of selected LiaSRₐₐ target genes in L. monocytogenes EGD exposed to cell wall-active antibiotics. L. monocytogenes EGD was exposed for 60 min to cell wall-active antibiotics which were added to the following concentrations: vancomycin, 30 μg ml⁻¹ (a); bacitracin, 700 μg ml⁻¹ (b); fosfomycin, 300 μg ml⁻¹ (c); polymyxin, 500 μg ml⁻¹ (d). The relative changes in transcription of lmo0955, lmo1966, lmo2486 and lmo2568 in the presence of antibiotics compared with a control culture in BHI were assessed by qRT-PCR. The ratios of the transcript amount detected in L. monocytogenes EGD grown in the presence of antibiotic compared with that of bacteria from the control culture (ratio, treated culture/untreated culture) are depicted. The indicated ratios represent the means of results of two qRT-PCR experiments performed in duplicate with cDNA which was reverse-transcribed from independent RNA preparations. The primer pairs generating the respective amplicons are listed in Supplementary Table S1. Error bars, SD.](image-url)
whether phosphorylation of LiaR\textsubscript{Lm} is required to activate transcription from LiaR\textsubscript{Lm}-dependent promoters, mutant \textit{L.m. liaR}\textsubscript{-D54Y} was constructed. This mutant expresses a mutated RR protein in which the aspartic acid residue at position 54 (D54) in the receiver domain is substituted by tyrosine. According to the RR receiver consensus sequence, D54 is predicted to be the site of phosphorylation of LiaR\textsubscript{Lm}, and it has been shown that mutagenesis of the corresponding residue (D55) in the LiaR orthologue VraR of \textit{S. aureus} abolishes receiver phosphorylation (Belcheva & Golemi-Kotra, 2008). As shown in Fig. 3(b, c), the transcription of selected target genes of LiaR\textsubscript{Lm} was similar in strains \textit{L.m. D liaR} and \textit{L.m. liaR}\textsubscript{-D54Y}, demonstrating that receiver phosphorylation of LiaR\textsubscript{Lm} is an absolute requirement for transcriptional induction of the LiaSR\textsubscript{Lm} regulon in response to cell wall-active antibiotics.

Since the gene products of the members of the LiaSR\textsubscript{Lm} regulon are likely to counteract alterations in cell envelope structure caused by antibiotic treatment, we tested the susceptibility of the mutants \textit{L.m. liaS} and \textit{L.m. liaR}\textsubscript{-D54Y} towards vancomycin, bacitracin, fosfomycin and polymyxin. However, in agar diffusion assays and when monitoring the growth curves of the bacteria in the presence of antibiotics in liquid culture, compared with the wild-type no differences in the susceptibility of the mutants towards these antibiotics were detected (data not shown).

\textbf{Promoter mapping defines a consensus binding site for the RR LiaR\textsubscript{Lm}}

To characterize the LiaR\textsubscript{Lm}-regulated promoters, the transcriptional start sites of selected target genes were mapped by 5'\textsuperscript{-}RACE. The 5' ends of the transcripts specific for lmo0047, lmo0955 and lmo2568 were mapped to positions -30, -57, -52, -169, -20 and -26 with respect to the translational start site. The -10 promoter elements corresponding to these transcriptional start sites are listed in Fig. 4. In the region upstream of the mapped promoters a highly conserved octanucleotide sequence motif, ATAnGACT, is located at a distance of 54–57 nt from the -10 box, which might constitute part of the RR binding site. This sequence motif is part of a region of 25 bp showing a lower overall degree of similarity, but harbouring the second half-site of a 7-2-7
inverted repeat sequence (Fig. 4) which is also present in the promoter regions of LiaR-dependent genes of Bacillus species (Jordan et al., 2006; Eldholm et al., 2010). In fact, in gel retardation experiments, binding of His$_6$-LiaR$_{Lm}$ to a 39 bp labelled DNA probe containing the ATAnGACT motif could be observed (Fig. 5) when 0.12 or 0.24 nmol of in vitro-phosphorylated protein was added to 20 fmol double-stranded DNA in the presence of 1 μg of the non-specific competitor poly-dIdC. The DNA probe used was derived from the upstream region of lmo2568. Addition of unlabelled probe to the binding reaction in a 20- or 100-fold molar excess abolished the band shift, indicating specific binding of the protein to the oligonucleotide (Fig. 5). His$_6$-LiaR$_{Lm}$ which was not in vitro-phosphorylated with acetyl phosphate prior to the binding reaction did not form a complex with the oligonucleotide probe (data not shown). A 35 bp DNA probe from the promoter region of the 16S rRNA gene of Helicobacter pylori whose chromosomal DNA also has a low GC content was used as a negative control, and no binding of His$_6$-LiaR$_{Lm}$ was observed (Fig. 5).

**Fig. 4.** LiaR$_{Lm}$-dependent promoters. The transcriptional start sites of several members of the LiaSR$_{Lm}$ regulon were mapped by 5'–RACE. The nucleotide representing the experimentally determined transcriptional start site is underlined. The alignment highlights a region of 25 nt in the upstream regions of the analysed genes that exhibits pronounced similarity. The upstream region of liaFlm (lmo1020) is included in the alignment, although the liaFlm promoter was not mapped experimentally. Nucleotides within the 25 bp region which are identical in more than half of the sequences are shown in upper-case type. The distance between the conserved 25 bp region and the putative −10 promoter element (italic type) is indicated (N$_x$). WebLogo (Crooks et al., 2004) representations of the position weight matrices derived from the sequences of the conserved 25 bp regions are shown above the alignment. The 7–2–7 inverted repeat, which has been proposed to represent the LiaR binding site in Bacillus species (Eldholm et al., 2010), is indicated by black arrows.

**Fig. 5.** Binding of His$_6$-LiaR$_{Lm}$ to the degenerate 7–2–7 inverted repeat sequence in the upstream region of LiaR$_{Lm}$-dependent genes. EMSA with His$_6$-LiaR$_{Lm}$ was performed on a 37 bp DNA probe derived from the upstream region of lmo2568 containing the degenerate 7–2–7 inverted repeat shown in Fig. 4. Lane 1, labelled DNA probe. In lanes 2–4, 0.06, 0.12 and 0.24 nmol His$_6$-LiaR$_{Lm}$ that was in vitro-phosphorylated with acetyl phosphate prior to the binding reaction was added. In lanes 6–9, a 20-fold (lanes 6 and 8) or 100-fold (lanes 7 and 9) molar excess of unlabelled DNA probe was added to binding reactions containing either 0.12 nmol (lanes 6 and 7) or 0.24 nmol (lanes 8 and 9) phosphorylated His$_6$-LiaR$_{Lm}$. In lane 5, a 35 bp DNA probe derived from the upstream sequence of the 16S rRNA gene of H. pylori was used as a negative control. The arrow on the right indicates the position of the protein–DNA complex.

**Derepression of the LiaSR$_{Lm}$ regulon in L.m. ΔliaS is due to the phosphorylation of LiaR$_{Lm}$ by acetyl phosphate**

In *B. subtilis*, LiaSR-dependent signal transduction is controlled via the membrane protein LiaF. Since deletion of liaF results in a derepressed LiaR-dependent genes, while they are downregulated in a liaS mutant, it is believed that an inhibitory interaction between LiaS and LiaF is relieved in the presence of the activating stimulus (Jordan et al., 2006).
et al., 2006). In contrast, in L. monocytogenes, deletion of the HK LiaS
Lm caused a derepression of the LiaSR
Lm regulon. Therefore, we hypothesized that LiaS
Lm is a bifunctional HK/phosphatase, whose phosphatase activity predominates in the absence of an activating stimulus. Under inducing conditions, the phosphatase activity would be switched off, leading to the phosphorylation of LiaR
Lm via the HK activity of LiaS
Lm. In analogy to the LiaSR system of B. subtilis, it is very likely that the switch between HK and phosphatase activity in LiaS
Lm is controlled via its interaction with LiaF
Lm. In this setting, the derepression of the LiaSR
Lm regulon in L.m. ∆liaS could be explained by the phosphorylation of LiaR
Lm via a non-cognate HK or via the low-molecular-mass phosphate donor acetyl phosphate, which in bacterial metabolism is produced via the pta/ack pathway from acetyl-CoA and acetate. To test this hypothesis, we tried to construct an in-frame deletion mutant of liaF
Lm. However, in several independent attempts to create the mutant via transformation of L. monocytogenes EGD with mutagenesis plasmid pLSV-1020, only wild-type bacteria could be recovered from the intermediate strain carrying a chromosomal integration of the mutagenesis plasmid, indicating that L. monocytogenes does not tolerate the lack of LiaF
Lm. As a control, mutant L.m. ∆liaR was transformed with pLSV-1020, yielding mutant L.m. ∆liaDliaR, which carries in-frame deletions of both liaF
Lm and liaR
Lm. As expected, according to qRT-PCR analysis, the transcript amounts of LiaR
Lm-dependent genes detected in the double mutant were as low as in the RR mutant L.m. ∆liaR (data not shown). These observations suggested that dysregulation of the LiaSR
Lm regulon in a liaF
Lm single mutant is lethal for L. monocytogenes.

To investigate the impact of acetyl phosphate on the transcriptional profile of strain L.m. ∆liaS, mutants L.m. ∆liaS/∆liaR-D54Y and L.m. ∆liaDpta/∆ackA were constructed, and the transcript amounts of selected members of the LiaSR
Lm regulon were determined via qRT-PCR analysis in comparison with the L. monocytogenes wild-type (Fig. 6). When L. monocytogenes was grown under inducing conditions in the presence of 10 µg vancomycin ml⁻¹, transcription of the LiaSR
Lm target genes was still considerably higher in L.m. ∆liaS than in the wild-type strain (Fig. 6a). Under the same conditions, in L.m. ∆liaS/∆liaR-D54Y, which lacks the HK LiaS
Lm and expresses a cognate RR deficient in receiver phosphorylation, a transcriptional profile similar to that of the RR mutant L.m. ∆liaR was observed (Fig. 6b). No derepression of LiaSR
Lm target genes was observed in mutant L.m. ∆liaDpta/∆ackA, which due to the lack of phosphotransacetylase and acetate kinase is unable to synthesize acetyl phosphate (Fig. 6c, d). As expected, their relative expression compared with the wild-type strain was lower when the bacteria were grown under inducing conditions (Fig. 6d). These data imply that derepression of the LiaSR
Lm regulon in the HK mutant L.m. ∆liaS is due to the phosphorylation of the RR LiaR
Lm by the metabolite acetyl phosphate.

DISCUSSION

The B. subtilis LiaFSR three-component system and its orthologues in the Firmicutes are involved in the cell envelope stress response, and constitute a discrete class of signal transduction systems, characterized by an IM-HK whose activity is controlled by a membrane-anchored negative regulator constituting the ‘third component’ (Mascher, 2006; Jordan et al., 2008). The regulons controlled by the LiaFSR orthologues have already been characterized by global transcriptional profiling in several Firmicutes, and it has been demonstrated that a common feature of this three-component system is positive auto-regulation (Kuroda et al., 2003; Martinez et al., 2007; Eldholm et al., 2010; Wolf et al., 2010), a feature also observed for L. monocytogenes liaFSR
Lm in this study. The regulons controlled by VraSR of S. aureus and CesSR of L. lactis mainly encode proteins involved in cell envelope biogenesis and proteins of unknown function (Kuroda et al., 2003; Martinez et al., 2007). The LiaFSR system of Streptococcus mutans has also been shown to regulate genes involved in cell envelope biogenesis and remodelling, as well as the genes encoding the membrane- and cell wall-associated proteases and chaperones FtsH and HtrA. However, in the case of this organism, no genome-wide analysis of the LiaFSR regulon has been reported (Suntharalingam et al., 2009). Furthermore, it has been observed that Streptococcus mutans LiaFSR regulates the expression of the essential TCS VicRK ( Tremblay et al., 2009), whose orthologues in Streptococcus pneumoniae and B. subtilis control genes involved in cell membrane and cell wall homeostasis ( Mohedano et al., 2005; Biscchia et al., 2007). In Streptococcus pneumoniae, 18 genes have been found to be positively regulated by the LiaFSR system, including the gene encoding phage shock protein C and the heat shock operon hrcA–grpE (Eldholm et al., 2010). In contrast, in B. subtilis, only three loci are subject to LiaR-dependent regulation, i.e. liaIGFSR, yycVZ–yhdA and yihE (Wolf et al., 2010; Mascher et al., 2004; Jordan et al., 2006). Recently, it has been suggested that liaIH represent the only in vivo-relevant LiaR target genes which upon cell envelope stress might orchestrate a response similar to the Pspa-dependent phage shock response of E. coli (Wolf et al., 2010). As already predicted by Jordan et al. (2006) based on the presence of a sequence motif with similarity to the B. subtilis LiaR binding site in the upstream region, we observed LiaR
Lm-dependent transcription of the liaIH orthologues lmo0954–lmo0955 in L. monocytogenes. Altogether, 27 genes were differentially expressed in an L. monocytogenes mutant lacking HK LiaS
Lm, which, in accordance with the role of the LiaSR orthologues in the cell envelope stress response, mainly encode proteins that are predicted to be membrane or extracytoplasmic proteins, including several components of ABC transporter systems. Interestingly, among the most strongly regulated target genes are three ORFs (lmo2484, lmo2485, lmo2567) which, like lia1, encode short membrane proteins encompassing 66 to 117 amino acids. Although, with the exception
of lmo0181 and lmo2015, differential regulation according to transcriptome analysis of members of all transcriptional units controlled by LiaS was confirmed by qRT-PCR, only a subset of 22 of these target genes was found to be differentially expressed in the RR mutant L. m. 

\[ LiaR \] when grown in the absence (Table 1) or presence of inducing agents (data not shown). Since the transcript amounts of the target genes were considerably higher in L. m. 

\[ LiaS \] than in the wild-type strain grown under inducing conditions (Fig. 6a), this discrepancy might be explained by the fact that in the presence of an increased level of 

\[ LiaR-Lm-P \] in L. m. 

\[ LiaS \], promoters harbouring a more divergent 

\[ LiaR-Lm \] binding motif may be transcribed which under ‘normal’ inducing conditions would not be targeted by 

\[ LiaR-Lm \].

The LiaSR-Lm TCS was induced in the presence of the antibiotics vancomycin and bacitracin, which interfere with the lipid II cycle, as has been observed with most of the other LiaSR orthologues studied so far (Mascher et al., 2004; Kuroda et al., 2003; Suntharalingam et al., 2009; Martinez et al., 2007). In addition, fosfomycin, which inhibits synthesis of UDP-N-acetyl-muramic acid, and polymyxin, which disrupts the structure of the cell membrane, induced transcription of the LiaSR-Lm regulon.
Activation in response to antibiotics which target very early steps in peptidoglycan synthesis is not a common feature of the LiaSR orthologues; however, activation of *B. subtilis* LiaSR and *S. aureus* VraSR in the presence of fosfomycin and D-cycloserine, respectively, has been reported (Mascher et al., 2004; Kuroda et al., 2003). Deletion of *liaSLm* or *liaRlm* did not alter the susceptibility of *L. monocytogenes* to the tested antibiotics. A similar observation has been made with liaSR deletion mutants of *B. subtilis* and *Streptococcus pneumoniae*, while in the case of *S. aureus*, *Streptococcus mutans* and *L. lactis*, deletion of the liaSR orthologues increases antibiotic susceptibility (Martinez et al., 2007; Eldholm et al., 2010; Mascher et al., 2004; Suntharalingam et al., 2009; Kuroda et al., 2003). Therefore, deletion of the liaSR orthologues seems to affect antibiotic susceptibility only in those bacteria whose genes directly involved in cell envelope synthesis are regulated by the TCS.

In the absence of agents causing perturbations of the cell envelope, the LiaSR system of *B. subtilis* is induced at the onset of stationary phase (Jordan et al., 2007). Growth phase-dependent transcription of liaSR has also been reported for *Streptococcus mutans*. However, in this case, lia gene expression is repressed in stationary phase relative to early and mid-exponential phase (Suntharalingam et al., 2009). In contrast, we did not observe a pronounced difference in the transcription of the liaF~SR locus in *L. monocytogenes* grown to the mid-exponential and stationary phases (data not shown).

Analysis of the mutant *L.m. liaR-D54Y* demonstrated that induction of transcription of LiaR~Lm~dependent promoters is strictly dependent on the phosphorylation of the RR. Mapping of the transcriptional start site of six target genes revealed the presence of a highly conserved octanucleotide motif, ATAnGACT, at a distance of approximately 50 bp from the −10 box. This motif forms the left half of a 7-2-7 inverted repeat, which was deduced from the upstream region of the *lia* orthologues of *Bacillus* and *Listeria* species, and which has been proposed to constitute the LiaR binding site (Jordan et al., 2006; Eldholm et al., 2010). Surprisingly, the right half of the proposed 7-2-7 inverted repeat is not well conserved in the LiaR~Lm~dependent *Listeria* promoters. In fact, specific binding of the phosphorylated RR LiaR~Lm~ to a short DNA probe containing the degenerate 7-2-7 inverted repeat sequence could be demonstrated by gel retardation experiments. In the case of lactococci and streptococci, the LiaR orthologues are predicted to bind to a 6-4-6 inverted repeat sequence, while VraR of *S. aureus* has been shown to bind to the sequence motif ACT(x)₅AGT, where x is any nucleotide and n may vary from 1 to 3 (Eldholm et al., 2010; Belcheva et al., 2009). Footprint experiments demonstrated that VraR binds to three distinct sites in the promoter of the vraSR locus which are centred at positions −76, −60 and −35 with respect to the transcriptional start site. Phosphorylation of VraR extended the binding to the −76 and −35 regions, which contain less well-conserved VraR binding motifs (Belcheva et al., 2009). Further experiments are required to precisely define the DNA-binding characteristics of LiaR~Lm~ and to elucidate the role of the poorly conserved right half-site of the degenerate 7-2-7 inverted repeat.

In *B. subtilis* and *Streptococcus mutans*, LiaF has been shown to act as an inhibitor of LiaSR-mediated signal transduction in the absence of an appropriate stimulus. Deletion of liaF causes the derepression of target genes under non-inducing conditions, while transcription is downregulated in liaR and liaS mutants (Jordan et al., 2006; Suntharalingam et al., 2009). In contrast, we observed a strong derepression of the LiaR~Lm~ regulon in the *Listeria* mutant lacking liaSLm with transcript amounts being even higher than in the wild-type strain exposed to the inducing agent vancomycin. Since derepression was strictly dependent on the phosphorylated RR LiaR~Lm~, these data suggested that LiaSLm is a bifunctional HK/phosphatase and that LiaR~Lm~ is efficiently phosphorylated in the absence of its cognate HK by an alternative phosphate donor. The phosphatase activity of LiaR~Lm~ towards LiaR~Lm~−P predominates in the absence of a stimulus, while it is switched off under inducing conditions, resulting in the accumulation of LiaR~Lm~−P. LiaR~Lm~−P did not accumulate in *in vitro* phosphorylation assays in the presence of a truncated LiaS~Lm~, lacking its N-terminal signalling domain (Fig. 1), corroborating the pronounced phosphatase activity of LiaS~Lm~. In fact, the phosphatase activity of the LiaS orthologue VraS of *S. aureus* towards its cognate phosphorylated RR has been reported (Belcheva & Golemi-Kotra, 2008). The archetype of a bifunctional HK is the well-studied NtrB protein that controls nitrogen assimilation in *E. coli* and other enteric bacteria. In NtrB, the catalytic ATPase (CA) domain inhibits the phosphatase activity towards NtrC~P that resides in the dimerization and histidine phosphorylation (DHP) domain (Kramer & Weiss, 1999; Jiang et al., 2000). Inhibition of the NtrB phosphatase activity is relieved by the binding of the PII protein to the CA domain (Jiang et al., 2000; Pioszak et al., 2000; Pioszak & Ninfa, 2003). Other bifunctional HKs, such as *E. coli* CpxA, do not rely on protein–protein interactions with a regulatory protein to control their phosphatase activity (Fleischer et al., 2007). The bifunctionality of an HK is considered to be an effective means to avoid cross-talk from other TCSs *in vivo* or from the accumulation of low-molecular-mass phosphodonor in the bacterial cell under certain growth conditions (Alves & Savageau, 2003; Laub & Goulia, 2007). This concept is sustained by the transcription profile of mutant *L.m. AliaS*, which we demonstrated to be due to the efficient phosphorylation of the RR LiaR~Lm~ by acetyl phosphate (Fig. 6). Evidence that acetyl phosphate accumulates in *L. monocytogenes* to levels sufficient for RR phosphorylation has already been provided by Guerrini et al. (2008b), who showed that deletion of the pta–ackA pathway impacts on the regulation of motility and chemotaxis via the orphan RR DegU. In analogy to the LiaFSR system of *B. subtilis*, we
propose that the activity state of LiaS_{Lm} is controlled via membrane-bound LiaF_{Lm}. We suggest that in the absence of an inducing signal, LiaF_{Lm} stimulates the phosphatase activity of LiaS_{Lm} towards LiaR_{Lm}−P, while the kinase activity of LiaS_{Lm} dominates when the interaction with the regulatory protein LiaF_{Lm} is relieved under inducing conditions. This model is based on the fact that we were unable to obtain a deletion mutant of liaF_{Lm} using L. monocytogenes EGD as the parent strain, while liaF_{Lm} could be knocked out in the RR mutant L.m. AliaR. We speculate that the uncontrolled HK activity of LiaS_{Lm} caused by the knockout of liaF_{Lm} would lead to highly elevated levels of LiaR_{Lm}−P, resulting in a massive expression of membrane-associated proteins which might be lethal for the cell. If by the interaction of LiaF_{Lm} with LiaS_{Lm} the phosphatase activity of LiaS_{Lm} was switched off in the presence of an inducing stimulus, the phenotype of a liaF_{Lm} knockout mutant would be expected to be similar to that of L.m. liaR-D54Y. It should be noted that apparently the phosphatase activity of LiaS_{Lm} is not completely switched off when Listeria is exposed to vancomycin, since under these conditions, compared with the wild-type strain, higher transcript amounts of target genes were detected in L.m. AliaS. In principle, a similar mechanism of control of phosphatase and HK activity is conceivable for LiaS of B. subtilis, assuming that LiaR is not subject to cross-talk from acetyl phosphate or that acetyl phosphate does not accumulate to significant levels under the growth conditions applied in the study of Jordan et al. (2006). In fact, for VraR, the only LiaR orthologue which has so far been biochemically characterized in vitro, 200-fold slower phosphorylation by acetyl phosphate than by the cognate HK VraS has been reported (Belcheva & Golemi-Kotra, 2008). The proposed model of signal transduction of the LiaFSR-like three-component systems is reminiscent of the essential YycFG TCS of B. subtilis that regulates cell wall turnover and cell division. The phosphorylation level of the RR YycF is negatively controlled by the formation of a complex of the HK YycG with its membrane-associated auxiliary proteins YycH and YycI via the interaction of their transmembrane helices (Szurmant et al., 2007, 2008). Interestingly, the orthologous HK WalK (VicK) of Streptococcus pneumoniae has been shown to possess phosphatase activity towards its cognate RR WalR−P (VicK−P), which requires an intact PAS domain (Gutu et al., 2010). However, the N-terminal domains of YycG and WalK_{Spn} differ, and orthologues of yycHI are not present in Streptococcus pneumoniae, suggesting a different mode of activity control for WalK_{Spn}.

In conclusion, we have demonstrated that LiaFSR_{Lm}-mediated signal transduction in L. monocytogenes relies on a finely tuned interplay between the HK and phosphatase activities of LiaS_{Lm}, which is presumably controlled by the membrane protein LiaF_{Lm}. The LiaS_{Lm}-dependent response to cell envelope perturbations then results in an extensive remodelling of the protein composition of the cytoplasmic membrane. It is tempting to speculate that some of the regulated membrane proteins contribute to a phase shock protein-like response orchestrated by LiaIH (Lmo0954–Lmo0955), as recently suggested by Wolf et al. (2010).

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REFERENCES


Gene autorepresses its own synthesis and is required for bacterial motility, virulence and biofilm formation. Mol Microbiol 70, 1342–1357.


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