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

Correspondence
Dagmar Beier
d.beier@biozentrum.
uni-wuerzburg.de

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

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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.

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 septicaemia and meningoenphalitis (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, which acts as an antagonist of the flagellar repressor MogR (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

[†]Present address: Chemisches und Veterinäruntersuchungsamt Stuttgart, Abteilung Mikrobiologie und Toxikologie, Schaflandstraße 3/2, 70736 Fellbach, Germany.

[‡]Present address: Medizinische Klinik und Poliklinik I, Universität Würzburg, Oberdürrbacher Str. 6, 97080 Würzburg, Germany.

Abbreviations: EMSA, electrophoretic mobility shift assay; HK, histidine kinase; IM-HK, intramembrane-sensing histidine kinase; qRT-PCR, quantitative real-time PCR; 5'-RACE, rapid amplification of 5' cDNA ends; RR, response regulator; TCS, two-component system.

The microarray data discussed in this paper are available from GEO under accession number GSE24107.

A supplementary table, showing oligonucleotides used in this study, is available with the online version of this paper.

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 *liaIHGFSR* 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 *yhcYZ-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_{Lm}. We report the characterization in *L. monocytogenes* of the LiaSR_{Lm} regulon, which consists mainly of genes encoding membrane-associated or

putative secreted proteins. We demonstrate that, as reported for other LiaSR orthologues, transcription of LiaR_{Lm} 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_{Lm}.

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* Δ 1022 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. Δ liaR* 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 μ g ml⁻¹. *E. coli* strains DH5 α , BL21 and M15 (pREP4) were grown in Luria–Bertani (LB) broth. When necessary, antibiotics were added to the following final concentrations: ampicillin, 100 μ g ml⁻¹; kanamycin, 50 μ g ml⁻¹; erythromycin, 300 μ g ml⁻¹.

Construction of *L. monocytogenes* mutants *L.m. Δ liaS*, *L.m. liaS, *L.m. liaR-D54Y*, *L.m. Δ liaS, liaR-D54Y*, *L.m. Δ liaF Δ liaR*, and *L.m. Δ liaS Δ pta Δ ackA*.** 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 (Wuenschel *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. Δ liaS* has an in-frame deletion of the region encoding amino acids 3–276 of the HK LiaS_{Lm}. To construct the knockout plasmid pLSV-1021, a 524 bp *Sall*–*NcoI* fragment PCR-amplified with primer pair 1021-1/1021-2 and derived from ORF lmo1020, and a 437 bp *NcoI*–*BamHI* fragment PCR-amplified with primer pair 1021-3/1021-4 and comprising the region encoding amino acids 277–352 of LiaS_{Lm} and amino acids 1–68 of LiaR_{Lm}, were cloned into pLSV1 vector DNA. The complemented strain *L.m. liaS** was obtained by transformation of *L.m. Δ liaS* with plasmid pLSV-1021c carrying a 1777 bp *Sall*–*BamHI* fragment PCR-amplified with primer pair 1021-1/1021-4 and comprising the *liaS*_{Lm} gene as well as 524 and 200 bp from its 5' and 3' flanking sequences, respectively. Mutant *L.m. liaR-D54Y* expresses a derivative of RR LiaR_{Lm} 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-1022D54Y, which was used to transform mutant *L.m. Δ liaR* (Williams *et al.*, 2005a). Mutant *L.m. Δ liaS, liaR-D54Y* was obtained by transformation of *L.m. liaR-D54Y* with suicide plasmid pLSV-1021. Knockout plasmid pLSV-1020 contained a 565 bp *EcoRI*–*XhoI* 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 *XhoI*–*BamHI* fragment comprising the last codon of lmo1020 and codons 1–220 of LiaS_{Lm} (amplified with primer pair lmo1020-3/lmo1020-4). pLSV-1020 was used to transform strain *L.m. Δ liaR* to yield the double mutant *L.m. Δ liaF Δ liaR*.

Knockout plasmids pLSV-pta and pLSV-ackA were used to construct deletions of *pta* and *ackA* in strain *L.m.* Δ *liaS* via 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 664 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_{Lm} and LiaS_{Lm}, and purification of fusion proteins. A 632 bp DNA fragment encoding the transmitter domain (amino acids 146–352) of the HK LiaS_{Lm} 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 plasmid 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_{Lm}, was selected by restriction of plasmid DNA with *EcoRI* and was designated pGEX-1021. To generate plasmid pQE-1022, a 666 bp DNA fragment encoding the RR LiaR_{Lm} 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 His₆ tag. The fusion protein GST–LiaS_{Lm} was produced in *E. coli* BL21 and purified by affinity chromatography on glutathione-Sepharose 4B, as described previously (Beier & Frank, 2000). His₆–LiaR_{Lm} was overproduced in *E. coli* M15 (pREP4) (Qiagen), and protein purification on Ni²⁺-nitrilotriacetic acid agarose columns (Qiagen) was carried out following the manufacturer's recommendations. The recombinant His₆–LiaR_{Lm} protein was stored in 50 mM Tris/HCl (pH 7.0), 150 mM NaCl, 500 mM KCl, 10 mM MgCl₂, 10 mM CaCl₂, 30 % (v/v) glycerol.

In vitro phosphorylation assays. *In vitro* phosphorylation assays providing 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 MgCl₂, 0.2 M ATP, 250 nM [γ -³³P]ATP (3000 Ci mmol⁻¹; 111 TBq mmol⁻¹)] 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_{Lm}~P to His₆–LiaR_{Lm} were performed on ice and were initiated by the addition of the RR protein to GST–LiaS_{Lm} that had been incubated for 15 min at 37 °C in reaction buffer containing 250 nM [γ -³²P]ATP. The reactions were stopped by the addition of sample buffer [60 mM Tris/HCl, pH 7.5, 50 mM Na₂EDTA, 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). RNA 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 $\Delta\Delta C_t$ 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 OD₆₀₀ 1.0. cDNA synthesis and hybridization of custom-made whole-genome microarrays containing 70mer oligodeoxyribonucleotides covering all ORFs of the *L. monocytogenes* EGD-e genome were performed essentially as described previously (Williams *et al.*, 2005b). Raw TIFF 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 labeling 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 His₆–LiaR_{Lm} was performed as described previously (Dietz *et al.*, 2002).

RESULTS

RR LiaR_{Lm} is phosphorylated by the HK LiaS_{Lm} *in vitro*

The genes *liaSR_{Lm}* belong to the transcriptional unit lmo1020–lmo1023, which also comprises a *liaF* orthologue (lmo1020) and a gene encoding a protein of unknown function (lmo1023). To investigate the interaction between the HK LiaS_{Lm} and the RR LiaR_{Lm}, *in vitro* phosphorylation assays with the purified recombinant proteins were performed. LiaS_{Lm} 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 cytoplasmic domain comprising amino acids 146–352 was fused to an N-terminal GST tag. The cognate RR LiaR_{Lm} was expressed with an N-terminal His₆ tag. Incubation of GST–LiaS_{Lm} in the presence of [γ -³³P]ATP resulted in the autophosphorylation of the HK (Fig. 1), while His₆–LiaR_{Lm} was not phosphorylated under these conditions (data not shown). When GST–LiaS_{Lm} and His₆–LiaR_{Lm} were combined under multiple turnover conditions, complete dephosphorylation of GST–LiaS_{Lm}~P was observed due to the phosphoryl group transfer to His₆–LiaR_{Lm}; however, the phosphorylated RR protein did not accumulate to significant levels (Fig. 1a). Phosphorylated His₆–LiaR_{Lm} could only be detected within the first 90 s of incubation on ice upon its

Fig. 1. Autophosphorylation of GST-LiaS_{Lm} and phosphotransfer to His₆-LiaR_{Lm}. (a) Reaction mixtures containing 1 μM GST-LiaS_{Lm} (lane 1) or 1 μM GST-LiaS_{Lm} and 6 μM His₆-LiaR_{Lm} (lane 4) were incubated in the presence of [γ -³²P]ATP for 15 min at 37 °C. The reactions were stopped by the addition of sample buffer and the reaction mixtures were analysed on a 12 % SDS-polyacrylamide gel. The position of phosphorylated GST-LiaS_{Lm} is indicated on the right. In lanes 2 and 3, GST-LiaS_{Lm} was combined under the same conditions with the recombinant *L. monocytogenes* RR protein His₆-DegU and a phosphorylation-deficient derivative of DegU, respectively. In contrast to the results reported by Kamp & Higgins (2009), we observed very weak phosphorylation of DegU by LiaS_{Lm} (lane 2). (b) His₆-LiaS_{Lm} (2.4 μM) was incubated for 15 min in the presence of [γ -³²P]ATP (lane 1), and then 0.77 μM (lanes 2, 3 and 4) or 2 μM (lanes 5, 6 and 7) His₆-LiaR_{Lm} was added. The reaction mixtures were incubated on ice and the reactions were stopped at the indicated time points. The reaction mixtures were analysed on a 12 % SDS-polyacrylamide gel. The positions of phosphorylated GST-LiaS_{Lm} and His₆-LiaR_{Lm} are indicated on the right.

addition to GST-LiaS_{Lm} that had been pre-treated with [γ -³²P]ATP for 15 min (Fig. 1b), indicating rapid hydrolysis of LiaR_{Lm}~P in the presence of LiaS_{Lm}.

Whole-genome transcriptional profiling of mutants *L.m.* Δ liaS and *L.m.* Δ liaR

To characterize the regulon controlled by the TCS LiaSR_{Lm}, the mutant *L.m.* Δ liaS carrying an in-frame deletion of the HK gene *liaS_{Lm}* was constructed. Whole-genome microarray analysis was performed with the mutant using a custom-made whole-genome microarray containing 70mer oligodeoxyribonucleotides 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.* Δ liaS 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.* Δ liaS. 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.* Δ liaS was confirmed (Table 1). Unexpectedly, transcriptome analysis of the RR deletion mutant *L.m.* Δ liaR, 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.* Δ liaS 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_{Lm}, LiaR_{Lm} acts as a transcriptional activator of the LiaSR_{Lm} regulon. To confirm that differential gene expression in *L.m.* Δ liaS was due to the deletion of the HK gene, *liaS* was reintroduced by allelic replacement into mutant *L.m.* Δ liaS to create the complemented strain *L.m.* *liaS*^{*}. The transcript amounts of selected target genes (lmo0047, lmo0955 and lmo2568) were compared in *L.m.* *liaS*^{*} and the wild-type strain via qRT-PCR, and proved to be similar (data not shown).

Transcription of LiaSR_{Lm} 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_{Lm} regulon to several antibiotics

Table 1. ORFs of the LiaSR_{Lm} regulon

Genes whose transcription according to microarray analysis differed more than twofold (ratio >2.0 or <0.5) in the *L. monocytogenes* HK mutant *L.m. ΔliaS* 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. ΔliaR* as compared with the wild-type strain EGD.

ORF	Function	Genome organization	Microarray ratio, <i>ΔliaS</i> /wild-type	qRT-PCR ratio, <i>ΔliaS</i> /wild-type	Microarray ratio, <i>ΔliaR</i> /wild-type
lmo0047	Unkown	Monocistronic	5.00	15.0	0.35
lmo0181	Unknown, similar to sugar ABC transporter (sugar-binding protein)		6.07	0.9	
lmo0193	Unknown	op 29 (lmo0193–0195)	3.33	11.4	0.43
lmo0194	ABC transporter, ATP-binding protein	op 29	3.39		0.41
lmo0195	Unknown, similar to membrane protein	op 29	3.07		0.39
lmo0954	<i>liaI</i>	op 149 (lmo0954–0955)	7.10		0.29
lmo0955	<i>liaH</i>	op 149	6.78	35.1	0.26
lmo1020	<i>liaF</i>	op 163 (lmo1018–1023)?	2.95	6.8	0.45
lmo1021	<i>liaS</i> , two-component sensor HK	op 163	2.23		
lmo1022	<i>liaR</i> , two-component RR	op 163	2.13		0.16
lmo1023	Unknown, similar to K ⁺ -uptake system	op 163	2.36		
lmo1636	Unknown, similar to ABC transporter (ATP-binding protein)	op 281 (lmo1635–1637)?	3.60	12.9	0.39
lmo1637	Unknown, similar to membrane protein	op 281	3.47		0.45
lmo1746	Unknown, similar to ABC transporter (permease)	op 306 (lmo1747–1741)?	2.29		
lmo1747	Unknown, similar to ABC transporter (ATP-binding protein)	op 306	2.16	6.1	
lmo1966	Unknown	op 356 (lmo1966–1967)	5.17	28.9	0.41
lmo1967	Unknown, similar to toxic ion resistance proteins	op 356	4.61		0.39
lmo2015	Unknown, similar to α-mannosidase		2.21	1.2	
lmo2207	Unknown		3.56	11.5	0.40
lmo2224	Unknown		2.62	7.0	
lmo2229	Unknown, similar to penicillin-binding protein	Monocistronic	2.42	3.9	
lmo2258	Unknown	Monocistronic	3.28	24.4	0.40
lmo2484	Unknown	op 441 (lmo2487–2484)	5.96		0.30
lmo2485	Unknown	op 441	6.24		0.24
lmo2486	Unknown	op 441	7.39	41.0	0.21
lmo2487	Unknown	op 441	8.50		0.23
lmo2567	Unknown		9.41		0.27
lmo2568	Unknown		11.69	296.5	0.25
lmo2745	Unknown, similar to ABC transporter (ATP-binding protein)	Monocistronic	2.41	6.3	0.48

which target the cell envelope. *L. monocytogenes* cells were grown to OD₆₀₀ 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 OD₆₀₀ 0.6 or after an additional 1 h of growth, depending on whether the effect of inhibitory or subinhibitory concen-

trations 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⁻¹, 300 μg ml⁻¹ and 700 μg ml⁻¹, respectively) resulted in a pronounced induction of transcription of the tested target genes (Fig. 2), with

bacitracin eliciting the strongest response. Bacitracin at a subinhibitory concentration ($100 \mu\text{g ml}^{-1}$) 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 ($0.5 \mu\text{g ml}^{-1}$), 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 ($20 \mu\text{g ml}^{-1}$) 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 ($500 \mu\text{g ml}^{-1}$) also activated the LiaSR_{Lm} 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 LiaSR_{Lm} target genes (data not shown).

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

Fig. 2. Expression analysis of selected LiaSR_{Lm} 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, $30 \mu\text{g ml}^{-1}$ (a); bacitracin, $700 \mu\text{g ml}^{-1}$ (b); fosfomycin, $300 \mu\text{g ml}^{-1}$ (c); polymyxin, $500 \mu\text{g ml}^{-1}$ (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.

Fig. 3. Expression analysis of selected LiaSR_{Lm} target genes in mutants *L.m. ΔliaR* and *L.m. liaR-D54Y*. (a) Mutant *L.m. ΔliaR* was exposed for 1 h to 10 μg vancomycin ml⁻¹. The relative changes in transcription of Imo0047, Imo0955 and Imo2568 in the presence of vancomycin compared with a control culture of *L.m. ΔliaR* grown in the absence of antibiotic were assessed by qRT-PCR. The ratios of the transcript amount detected in *L.m. ΔliaR* grown in the presence of vancomycin compared with that of bacteria from the

control culture (ratio, treated culture/untreated culture) are depicted. (b, c) *L. monocytogenes* EGD and mutants *L.m. ΔliaR* and *L.m. liaR-D54Y* were cultivated in the presence of 10 μg vancomycin ml⁻¹. The relative changes in transcription of Imo0047, Imo0955 and Imo2568 in the mutant strains compared with the wild-type strain were analysed by qRT-PCR. The ratios of transcript amount detected in mutant *L.m. ΔliaR* (b) and mutant *L.m. liaR-D54Y* (c) compared with that of the wild-type strain (ratio, mutant/wild-type) are shown. 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. Error bars, SD.

whether phosphorylation of LiaR_{Lm} is required to activate transcription from LiaR_{Lm}-dependent promoters, mutant *L.m. liaR-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_{Lm} and it has been shown that mutagenesis of the corresponding residue (D55) in the LiaR orthologue VraR of *S. aureus* abolishes receiver phosphorylation (Belcheva & Golemi-Kotra, 2008). As shown in Fig. 3(b, c), the transcription of selected target genes of LiaR_{Lm} was similar in strains *L.m. ΔliaR* and *L.m. liaR-D54Y*, demonstrating that receiver phosphorylation of LiaR_{Lm} is an absolute requirement for transcriptional induction of the LiaSR_{Lm} regulon in response to cell wall-active antibiotics.

Since the gene products of the members of the LiaSR_{Lm} regulon are likely to counteract alterations in cell envelope structure caused by antibiotic treatment, we tested the susceptibility of the mutants *L.m. ΔliaS* and *L.m. ΔliaR* 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).

Promoter mapping defines a consensus binding site for the RR LiaR_{Lm}

To characterize the LiaR_{Lm}-regulated promoters, the transcriptional start sites of selected target genes were mapped by 5'-RACE. The 5' ends of the transcripts specific for Imo0047, Imo0193, Imo0954, Imo1966, Imo2258 and Imo2568 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

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 *liaF_{Lm}* (lmo1020) is included in the alignment, although the *liaF_{Lm}* 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.

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 His₆-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). His₆-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 His₆-LiaR_{Lm} was observed (Fig. 5).

Derepression of the LiaSR_{Lm} regulon in *L.m.* Δ*liaS* 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 derepression of 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

Fig. 5. Binding of His₆-LiaR_{Lm} to the degenerate 7-2-7 inverted repeat sequence in the upstream region of LiaR_{Lm}-dependent genes. EMSA with His₆-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 His₆-LiaR_{Lm} that was *in vitro*-phosphorylated with acetyl phosphate prior to the binding reaction was added. In lanes 5–9, a 20-fold (lanes 5 and 6) or 100-fold (lanes 7 and 8) molar excess of unlabelled DNA probe was added to binding reactions containing either 0.12 nmol (lanes 5 and 6) or 0.24 nmol (lanes 7 and 8) phosphorylated His₆-LiaR_{Lm}. In lane 9, 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.

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.* Δ 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/ackA* 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.* Δ liaR was transformed with pLSV-1020, yielding mutant *L.m.* Δ liaF Δ liaR, 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.* Δ 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.* Δ liaS, mutants *L.m.* Δ liaS, *liaR*-D54Y and *L.m.* Δ liaS Δ pta Δ 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 μ g vancomycin ml⁻¹, transcription of the LiaSR_{Lm} target genes was still considerably higher in *L.m.* Δ liaS than in the wild-type strain (Fig. 6a). Under the same conditions, in *L.m.* Δ 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.* Δ liaR was observed (Fig. 6b). No derepression of LiaSR_{Lm} target genes was observed in mutant *L.m.* Δ liaS Δ pta Δ 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.* Δ 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; Martínez *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; Martínez *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; Bisicchia *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. *liaIHGFSR*, *yhcYZ-yhdA* and *ydhE* (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 *liaI*, encode short membrane proteins encompassing 66 to 117 amino acids. Although, with the exception

Fig. 6. qRT-PCR analysis of mutants *L.m. ΔliaS*, *L.m. ΔliaS, liaR-D54Y* and *L.m. ΔliaSΔptaΔackA*. (a) Mutant *L.m. ΔliaS* and the wild-type strain *L. monocytogenes* EGD were exposed to 10 µg vancomycin ml⁻¹. Transcription of the LiaSR_{Lm} target genes lmo0047, lmo0955, lmo2486 and lmo2568 was analysed via qRT-PCR, and the ratios of the transcript amount detected in *L.m. ΔliaS* compared with that of the wild-type strain (ratio, mutant/wild-type) are depicted. (b) The transcript amounts of lmo0047 and lmo0955 were determined via qRT-PCR in mutant *L.m. ΔliaS, liaR-D54Y* and the wild-type strain *L. monocytogenes* EGD, which were exposed to 10 µg vancomycin ml⁻¹. The ratios of the transcript amount detected in *L.m. ΔliaS, liaR-D54Y* compared with that of the wild-type strain (ratio, mutant/wild-type) are depicted. (c, d) Mutant *L.m. ΔliaSΔptaΔackA* and the wild-type strain *L. monocytogenes* EGD were cultivated in the absence (c) and presence (d) of 10 µg vancomycin ml⁻¹. Differential transcription of the LiaSR_{Lm} target genes lmo0047, lmo0955 and lmo2568 in the mutant and the wild-type strain was analysed, and the ratios of the transcript amount detected in *L.m. ΔliaSΔptaΔackA* compared with that of the wild-type strain (ratio, mutant/wild-type) 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. Error bars, SD.

of lmo0181 and lmo2015, differential regulation according to transcriptome analysis of members of all transcriptional units controlled by LiaS_{Lm} 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. ΔliaR* 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. ΔliaS* 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. ΔliaS*, 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; Martínez *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 *liaS_{Lm}* or *liaR_{Lm}* 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 (Martínez *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 *liaFSR_{Lm}* 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 *liaI* 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)_nAGT, 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 LiaSR_{Lm} regulon in the *Listeria* mutant lacking *liaS_{Lm}*, 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 LiaS_{Lm} 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 LiaS_{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 phosphodonors in the bacterial cell under certain growth conditions (Alves & Savageau, 2003; Laub & Goulian, 2007). This concept is sustained by the transcription profile of mutant *L.m. ΔliaS*, 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 Gueriri *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. ΔliaR*. 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. ΔliaS*. 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 (VicR~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 LiaSR_{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 phage shock protein-like response orchestrated by LiaIH (Lmo0954–Lmo0955), as recently suggested by Wolf *et al.* (2010).

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