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Genome-wide transcriptional profiling of the cell envelope stress response and the role of LisRK and CesRK in *Listeria monocytogenes*

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The Gram-positive bacterium *Listeria monocytogenes* is widely distributed in the environment and capable of causing food-borne infections in susceptible individuals. In this study, we investigated the cell envelope stress response in *L. monocytogenes*. Whole-genome transcriptional profiling was performed to investigate the response upon exposure to the cell wall antibiotic cefuroxime. Differential expression (at least twofold) of 558 genes was observed, corresponding to 20 % of the *L. monocytogenes* genome. The majority of genes that were strongly induced by cefuroxime exposure have cell-envelope-related functions, including the *dlt* operon and the gene encoding penicillin-binding protein PBPD2. A large overlap was observed between the cefuroxime stimulon and genes known to be induced in *L. monocytogenes* in blood and during intracellular infection, indicating that the cell envelope stress response is active at various stages of the infectious process. We analysed the roles of the two-component systems LisRK and CesRK in the cell envelope response, showing that activation of the most highly cefuroxime-induced genes was LisR- and CesR-dependent. In addition, multiple VirRS- and LiaSR-regulated genes were found to be induced in response to cefuroxime exposure. In total, 53 % of the genes upregulated at least fourfold by cefuroxime exposure are under positive control by one of the four two-component systems. Using genetic analyses, we showed that several genes of the cefuroxime stimulon contribute to the innate resistance of *L. monocytogenes* to cefuroxime and tolerance to other cell-envelope-perturbing conditions. Collectively, these findings demonstrate central roles for two-component systems in orchestrating the cell envelope stress response in *L. monocytogenes*.

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INTRODUCTION

Listeria monocytogenes is a Gram-positive, food-borne intracellular pathogen, causing life-threatening infections in susceptible individuals (Vázquez-Boland *et al.*, 2001). *L. monocytogenes* is able to survive and propagate within many different environments, including soil, food processing environments, the gastrointestinal tract and the cytosol of mammalian cells. In order to adapt and multiply within such diverse and changing surroundings, *L. monocytogenes* must continuously monitor and respond to environmental signals by means of complex gene regulatory networks that coordinate the expression of specific sets of genes supporting bacterial survival and growth. The genome of *L. monocytogenes* encodes 14 two-component signal transduction systems and a single orphan response regulator which are thought to contribute to the coordinated response to various

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Abbreviations: CAMP, cationic antimicrobial peptide; ECF, extracytoplasmic function; qRT-PCR, quantitative real-time PCR; TCS, two-component system.

The microarrays used in this study were generated and designed as described by van der Veen *et al.* (2010) (GEO Platform no. GPL14687) and the microarray data from this study have been deposited in the GEO database with accession no. GSE32913.

Three supplementary figures and five supplementary tables are available with the online version of this paper.

signals from the surroundings (Glaser *et al.*, 2001). Typical two-component systems (TCSs) consist of a membrane-bound sensor histidine kinase (HK) and its cognate cytoplasmic transcriptional regulator, the response regulator, which is usually kept in its inactive state in the absence of an inducing signal (Parkinson, 1993). When exposed to a specific stimulus from the environment, a histidine residue in the HK becomes autophosphorylated. The phosphoryl group is then transferred from the C-terminal transmitter domain of the HK to an aspartic acid residue in the N-terminal receiver domain of the response regulator, resulting in transcriptional activation or repression of specific target genes (Parkinson, 1993).

In *L. monocytogenes*, the TCSs LisRK and CesRK have been analysed with respect to their roles in stress tolerance and virulence. Both TCSs mediate ethanol sensitivity and β-lactam resistance, in particular to the cephalosporins, and mutant strains lacking functional LisRK or CesRK systems are less virulent in mice than their parent wild-type stains (Cotter *et al.*, 1999, 2002; Kallipolitis *et al.*, 2003; Kallipolitis & Ingmer, 2001). Furthermore, a *lisK* deletion mutant displays a growth-phase-dependent response to acidic conditions and is less tolerant to osmotic stress but more resistant to the lantibiotic nisin (Cotter *et al.*, 1999, 2002; Sleator & Hill, 2005). LisRK has been shown to be a positive regulator of five genes, including *htrA* (*lmo0292*), encoding the serine protease HtrA, *lmo1021*, encoding the HK of the TCS LiaSR, and *lmo2229*, encoding the penicillin-binding protein PBPA2 (Cotter *et al.*, 2002; Gottschalk *et al.*, 2008; Stack *et al.*, 2005). Transcription of the LisR-regulated gene *lmo2210*, encoding a protein of unknown function, is induced by subinhibitory concentrations of ethanol and the cephalosporin cefuroxime (Gottschalk *et al.*, 2008). The TCS CesRK has been shown to stimulate transcription of four genes in response to ethanol and cefuroxime exposure, including *lmo2812*, *lmo0443* and *lmo1416*, encoding the penicillin-binding protein PBPD2, a LytR-like regulatory protein and a putative transporter contributing to β-lactam resistance and ethanol susceptibility, respectively (Gottschalk *et al.*, 2008; Kallipolitis *et al.*, 2003). Thus, both LisRK and CesRK appear to be involved in sensing and responding to cell-envelope-damaging agents.

The integrity of the Gram-positive cell envelope, consisting of a cytoplasmic membrane surrounded by a thick cell wall, can be threatened by a number of cell-envelope-active compounds, including cell wall antibiotics and detergents, such as SDS and bile. Furthermore, general stress conditions (i.e. osmotic stress, heat, acid, etc.) have been shown to affect the integrity of the cell envelope (Jordan *et al.*, 2008). In general, the bacterial cell-envelope-stress response may be defined as those signal-transduction regulatory systems, as well as their regulons, that act to sense and respond to cell wall antibiotics and other cell-envelope-damaging conditions (Jordan *et al.*, 2008). In addition to LisRK and CesRK, the TCSs LiaSR and VirRS have been linked to the cell-envelope-stress response in *L. monocytogenes* (Fritsch *et al.*, 2011; Mandin *et al.*, 2005). LiaSR belongs to a conserved family of TCS which senses cell envelope perturbations caused by cell-wall-active antibiotics (Fritsch *et al.*, 2011; Jordan *et al.*, 2008). The LiaSR regulon in *L. monocytogenes* comprises multiple genes encoding membrane-associated and extracytoplasmic proteins, suggesting that activation of the LiaSR regulon results in remodelling of the cell envelope (Fritsch *et al.*, 2011). VirRS controls the expression of the *dlt* operon, involved in alanylation of the lipoteichoic acids in the cell envelope, and *mprF*, conferring resistance to cationic antimicrobial peptides (CAMPs) (Abachin *et al.*, 2002; Mandin *et al.*, 2005; Thedieck *et al.*, 2006).

In this report, we present a whole-genome transcriptional profiling of the response of *L. monocytogenes* to the β-lactam antibiotic cefuroxime, which disrupts the biosynthesis of the peptidoglycan layer by mimicking the peptide end of muropeptides thereby inhibiting the final transpeptidation reaction in peptidoglycan assembly. Finally, we investigated the roles of the TCSs LisRK and CesRK in the cell-envelope-stress response. Our data show that cell envelope stress stimulates the expression of multiple LisR- and CesR-dependent genes that contribute to the innate resistance of *L. monocytogenes* against cefuroxime as well as other cell-envelope-perturbing conditions. Our study demonstrates that approximately half of the genes induced at least fourfold upon cefuroxime exposure are activated by one of the four cell-envelope-related TCSs in *L. monocytogenes*: LisRK, CesRK, VirRS and LiaSR.

METHODS

Bacterial strains, plasmids and media. Wild-type *L. monocytogenes* serotype 1/2c strain LO28 (Vazquez-Boland *et al.*, 1992) and mutant derivatives were grown at 37 °C, 180 r.p.m., in brain heart infusion broth (BHI, Oxoid). Mutant derivatives of LO28 carrying in-frame deletions in *cesR*, *lisR*, *lmo1416*, *lmo2210* and *lmo2812* were constructed as described previously (Gottschalk *et al.*, 2008; Kallipolitis *et al.*, 2003). When required, 5 μg erythromycin ml^{-1} was added to the medium. *Escherichia coli* TOP10 (Invitrogen) was grown at 37 °C, 180 r.p.m., in Luria–Bertani medium (LB), and when required 150 μg erythromycin ml^{-1} was added.

Construction of in-frame deletion mutants. For construction of mutant strains carrying in-frame deletions in *lmo1037*, *lmo1518*, *lmo2442*, *lmo2522* or *lmo2568*, DNA fragments containing the flanking 5′- and 3′-regions of the gene of interest were generated by PCR amplification (29 cycles of 94 °C for 3 min, 50 °C for 1 min, 68 °C for 1 min; then 68 °C for 3 min and a 4 °C hold) using two sets of primers for each gene: Primer A and B and Primer C and D (Supplementary Table S1, available with the online version of this paper). PCR fragments carrying the in-frame deletions were amplified using Primers A and D with the 5′- and 3′-flanking DNA fragments used as template. The DNA fragments were inserted into the temperature sensitive shuttle vector, pAUL-A (Chakraborty *et al.*, 1992). Integration, allelic exchange and verification of in-frame deletions were performed as previously described (Christiansen *et al.*, 2004).

Isolation of total RNA, labelling, hybridization, imaging and microarray analysis. Overnight pre-cultures of LO28 wild-type, LO28Δ*lisR* and LO28Δ*cesR* mutant strains were diluted 100-fold in liquid medium and incubated until early exponential growth phase was reached (OD_{600} 0.35). Cefuroxime was added to a final concentration

of 4 μg ml^{-1}. Prior to (t_0) and 1 h after the addition (t_{60}), 20 ml samples were pelleted (Eppendorf centrifuge 5804R, motor type F-34-6-38, 10 000 r.p.m.) at 4 °C for 30 s. After decanting the supernatant, pellets were dissolved in 1 ml TRI-reagent (Ambion) and stored at −80 °C until total RNA isolation. Total RNA extraction, cDNA synthesis, labelling, microarray hybridization and scanning were performed as described previously (Mols *et al.*, 2010). The microarray experiments were performed in two independent biological replicates, with several technical replicates, where the replicate was performed with the dyes swapped (Mols *et al.*, 2010). The microarrays used in this study were generated and designed as described by van der Veen *et al.* (2010) (GEO Platform no. GPL14687).

Data analysis and validation. Data analysis was performed following the protocol described previously by Mols *et al.* (2010) using the web-based Vampire microarray suite (Hsiao *et al.*, 2005). Cut-off values of twofold for upregulated genes and 0.5-fold for downregulated genes were set as an additional threshold for relevant significant differential expression. Validation of the microarray was performed by plotting the log_2-transformed expression ratios obtained from the microarray data with log_2-transformed expression ratio obtained by quantitative real-time PCR. The microarray data from this study have been deposited in the GEO database (GEO Series no. GSE32913).

Quantitative real-time PCR (qRT-PCR). Cells (OD_{600} 0.35) were exposed to 4 μg cefuroxime ml^{-1}, 0.1 μg ampicillin ml^{-1}, 5 % ethanol, 0.08 % bile salts or 8 % sodium chloride, for 1 h. Unstressed cultures were used as controls. Total RNA was extracted as described previously (Nielsen *et al.*, 2010). Superscript III reverse transcriptase (Invitrogen) was used to synthesize cDNA using DNase-treated total RNA. qRT-PCR was performed using SYBR Green PCR Master Mix (Fermentas) and specific primer sets for the genes of interest (Supplementary Table S1). The primer sets, designed using primer3 (http://frodo.wi.mit.edu/primer3/), generated amplicons with a length of about 100 bp (Supplementary Table S1). A standard curve was generated for each primer using both genomic DNA and cDNA; pure water was used as negative control. Reactions were run on a MX3000 quantitative PCR thermocycler (Stratagene) with an initial step at 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. The data obtained were analysed using the 'Relative Expression Software Tool – Multiple Condition Solver' (Pfaffl, 2001; Pfaffl *et al.*, 2002). 16S-rRNA, *tpi* and *rpoB* were used as reference genes. RNA from three independent biological experiments was analysed in duplicates.

Computational search for LisR and CesR binding sites. Genes selected for binding-site analyses were significantly upregulated (fold change at least twofold) in wild-type LO28 in response to cefuroxime exposure and showed no significant upregulation in the Δ*cesR* or Δ*lisR* mutant strains. The 400 bp regions located upstream from the translational start sites of CesR-regulated genes were searched for sequences matching the CesR responsive element AATCTTAA (Gottschalk *et al.*, 2008) using the 'Search pattern' program available at the Listilist Web Server (http://genolist.pasteur.fr/ListiList/index.html). Putative CesR binding sites were aligned with the previously defined CesR binding motif using CLUSTAL_X (Larkin *et al.*, 2007). The 400 bp regions located upstream from the LisR-regulated genes were analysed for conserved motifs using the MEME program (Bailey & Elkan, 1994). The MEME search criteria were set at a minimal motif length of 6 nt and a maximal length of 35 nt.

Determination of MIC and stress tolerance assays. MIC was determined using E-tests calibrated with a MIC scale in μg ml^{-1} (bioMériux). Overnight cultures were spread on Müller–Hinton agar plates supplemented with 5 % sheep blood. E-tests supplied with cefuroxime, ampicillin and gentamicin were applied and plates were incubated at 37 °C. MIC values were determined after 24 h of incubation. For stress-tolerance assays, overnight cultures were diluted 1000-fold into BHI adjusted with cefuroxime (4 μg ml^{-1}), sodium chloride (8 %), SDS (0.1 %) or bile salts (0.08 %; DIFCO, bile salts no. 3). Bacterial growth was monitored for 30 h by measuring OD_{600} in a Shimadzu UV mini 1240 spectrophotometer. Three independent biological experiments were performed.

Statistical analyses. The data obtained in qRT-PCR and stress-tolerance experiments were analysed using Student's *t*-test. The differences reported were statistically significant with at least 95 % confidence.

RESULTS

Global gene expression analysis of the response to cefuroxime exposure

The response of the wild-type strain *L. monocytogenes* LO28 to a subinhibitory concentration (4 μg ml^{-1}) of cefuroxime was analysed by whole-genome expression profiling of exponentially growing wild-type cells harvested prior to (t_0) and 1 h after (t_{60}) the addition of cefuroxime. Within this time frame, the growth of LO28 in the presence of 4 μg cefuroxime ml^{-1} was similar to the growth of an unstressed culture (see Supplementary Fig. S1). Under these conditions, 558 genes showed at least twofold significant differential expression, corresponding to 20 % of its genome, with 284 genes being induced and 274 being repressed (Supplementary Tables S2 and S3). The microarray data of 18 upregulated and 12 downregulated genes was validated by qRT-PCR analysis (Supplementary Fig. S2 and Supplementary Tables S2 and S3). Considering that some of the differential effects observed may be the outcome of the 1 h induction time (i.e. t_0 versus t_{60}) rather than the stress condition applied (i.e. cefuroxime exposure), we decided to focus on the most highly upregulated or downregulated genes (i.e. at least fourfold regulation). As will be demonstrated later, no growth-related effects were observed for nine of the most highly upregulated genes (Fig. 1, described below).

Approximately half of the 73 genes found to be upregulated at least fourfold by cefuroxime exposure are predicted to encode proteins with cell-envelope-related functions, such as the Dlt-system and alternative penicillin-binding proteins (PBPs) (Supplementary Table S2). The *dlt*-gene cluster was found to be upregulated up to sixfold by cefuroxime exposure. The Dlt-system constitutes a core element in the Gram-positive cell envelope stress response (Jordan *et al.*, 2008) and is responsible for the incorporation of D-alanine into the cell-wall-associated teichoic acids, thereby lowering the overall negative charge of the cell envelope. In *L. monocytogenes*, the D-alanylation of teichoic acids is important for virulence, as the net charge of the surface has great influence on the interaction with other compounds and proteins (Abachin *et al.*, 2002). A second important element in the Gram-positive cell envelope stress response is the induction of alternative PBPs catalysing the final steps of cell wall biosynthesis (Jordan *et al.*, 2008). Our transcriptional analysis revealed that *lmo2812*, encoding PBPD2, was upregulated 12-fold by cefuroxime exposure (Supplementary

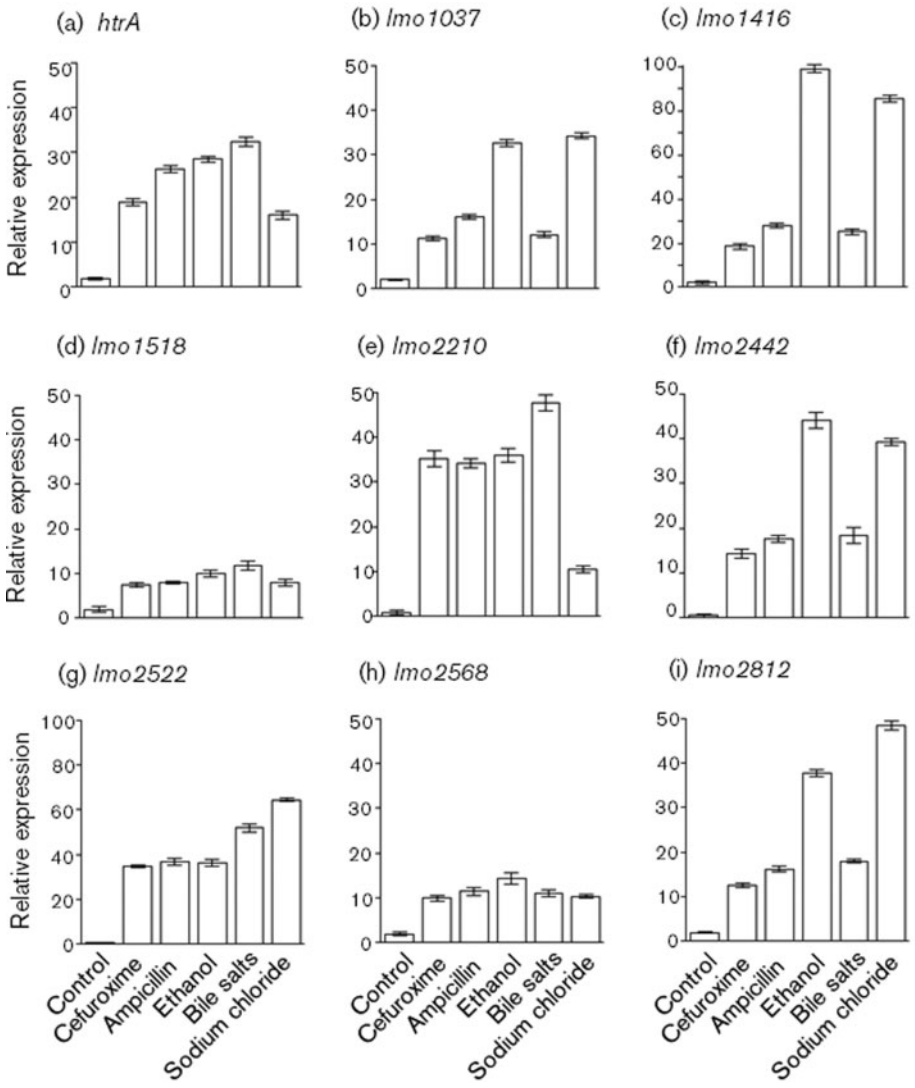

Fig. 1. The effect of various stress agents on the relative expression of selected genes highly induced by exposure to cefuroxime. Relative expression ratios tested by qRT-PCR analysis on total RNA isolated before and after 1 h exposure to 4 μg cefuroxime ml^{-1}, 0.1 μg ampicillin ml^{-1}, 5 % ethanol, 0.08 % bile salts or 8 % sodium chloride. Unstressed cultures were used as controls. Results are shown for the following genes: (a) *htrA*, (b) *lmo1037*, (c) *lmo1416*, (d) *lmo1518*, (e) *lmo2210*, (f) *lmo2442*, (g) *lmo2522*, (h) *lmo2568* and (i) *lmo2812*. Error bars represent the SDs of three independent experiments performed in duplicate. For all nine genes tested, the results for the stressed conditions were significantly different to results for the unstressed conditions ($P<0.001$).

Table S2). PBPD2 is a low-molecular-mass PBP displaying DD-carboxypeptidase activity that most probably participates in the late stages of peptidoglycan synthesis (Korsak *et al.*, 2010). In addition, minor effects on expression (two- to threefold) were observed for genes encoding the four high-molecular-mass PBPs PBPA1 (*lmo1892*), PBPA2 (*lmo2229*), PBPB1 (*lmo1438*) and PBPB3 (*lmo0441*) (Supplementary Table S2).

The response to cefuroxime exposure also includes an induced expression of stress related genes. The *ctc* gene (*lmo0211*) plays a protective role during osmotic stress and was found to be upregulated 5.5-fold by cefuroxime exposure (Gardan *et al.*, 2003). Furthermore, the expression of the *htrA* gene (*lmo0292*) encoding the serine protease HtrA, was induced 18-fold by cefuroxime exposure, corresponding to the third most upregulated gene in this study (Supplementary Table S2). HtrA is important for growth under stress conditions and contributes to virulence in *L. monocytogenes* (Stack *et al.*, 2005; Wilson *et al.*, 2006; Wonderling *et al.*, 2004).

Secreted and cell-envelope-associated proteins in *L. monocytogenes* are of great interest due to their putative roles in

adhesion, invasion of host cells and interaction with the immune system (Bierne & Cossart, 2007). In response to the presence of cefuroxime, *lmo2522* was found to be the most highly upregulated gene in *L. monocytogenes* (34-fold induction, Supplementary Table S2). *lmo2522* encodes a putative cell wall-binding protein containing a LysM domain, which is thought to be a general peptidoglycan binding module (Bierne & Cossart, 2007). *lmo2714*, encoding a LPXTG surface protein, was induced more than sixfold by cefuroxime exposure (Supplementary Table S2). LPXTG surface proteins, including the well-characterized internalin InlA, are characterized by being directly linked to the peptidoglycan. Interestingly, both *lmo2522* and *lmo2714* are induced during *in vivo* infection, and *lmo2714* has been shown to contribute to pathogenesis in mice (Camejo *et al.*, 2009). Furthermore, we noticed that the *lmo2120* gene was upregulated 4.4-fold by cefuroxime exposure (Supplementary Table S2). This gene encodes the di-adenylate cyclase DacA which activates the host cytosolic surveillance pathway during infection (Woodward *et al.*, 2010).

When comparing our data with the results of other studies employing whole genome transcriptional profiling, we observed a large overlap between the genes induced at least fivefold by cefuroxime exposure and the set of genes shown to be induced when *L. monocytogenes* is situated in blood (21 of 32 genes) and during intracellular infection in mammalian cells (18 of 32 genes) (Supplementary Table S4) (Chatterjee *et al.*, 2006; Joseph *et al.*, 2006; Toledo-Arana *et al.*, 2009). This supports the idea that *L. monocytogenes* is experiencing cell-envelope stress during infection of a host organism. We also observed a large overlap between the genes downregulated at least fivefold by cefuroxime exposure and the genes shown to be repressed when *L. monocytogenes* is exposed to mammalian bile, including the PrfA-regulon (60 of 110 genes, see Supplementary Table S5) (Milohanic *et al.*, 2003; Quillin *et al.*, 2011). The PrfA-activated virulence factors, encoded by *plcA*, *plcB*, *hly* and *actA*, are required for intracellular invasion and multiplication (de las Heras *et al.*, 2011). The expression of these genes was downregulated up to eightfold, whereas the *prfA* gene itself remained largely unaffected by cefuroxime exposure (Table 1). In addition, several PrfA-regulated genes located outside of the pathogenicity island LIPI-1 were downregulated by cefuroxime exposure as well, including *uhpT*, encoding a specific hexose-phosphate transporter, and *inlA*, *inlB* and *inlC*, which encode *Listeria*-specific internalins (Table 1). These observations show that the presence of cefuroxime leads to downregulation of the PrfA regulon.

Genome-wide analysis of the roles of LisRK and CesRK in cell envelope stress response

Signal-transducing regulatory systems, such as alternative sigma factors and TCSs that sense and respond to cell-envelope-perturbing conditions, represent a highly conserved element of the Gram-positive cell-envelope-stress response (Jordan *et al.*, 2008). Interestingly, the genes encoding the LisRK two-component system were found to be induced 4.17-fold (*lisR*) and 3.93-fold (*lisK*), respectively (Supplementary Table S2). In addition, four genes previously shown to be affected by LisRK (*htrA*, *lmo2210*, *lmo2229* and *lmo2487*) were highly upregulated in response to cefuroxime exposure (Supplementary Table S2) (Cotter *et al.*, 2002; Gottschalk *et al.*, 2008; Stack *et al.*, 2005). Our transcriptional analysis also showed that the CesRK-regulated genes *lmo0443*, *lmo1416*, *lmo2420* and *lmo2812* were highly upregulated by cefuroxime exposure (Supplementary Table S2) (Gottschalk *et al.*, 2008; Kallipolitis *et al.*, 2003). In order to gain information about the roles of LisRK and CesRK in the cell envelope stress response, we performed transcriptome analyses of the LO28Δ*lisR* and LO28Δ*cesR* mutant strains. Importantly, the whole-genome transcriptional profiling of the mutant strains, sampled before and 1 h after the addition of a subinhibitory concentration of cefuroxime (4 μg ml^{-1}), was performed in parallel with the wild-type strain, thus

Table 1. Downregulation of PrfA-regulated virulence genes in wild-type LO28 in response to the presence of 4 μg cefuroxime ml^{-1}

Gene	Name	Protein description	Microarray ratio*
lmo0200	*prfA*	Listeriolysin positive regulatory protein	1.39
lmo0433	*inlA*	Internalin A	6.28
lmo0434	*inlB*	Internalin B	4.09
lmo1786	*inlC*	Internalin C	2.03
lmo0201	*plcA*	Phosphatidylinositol-specific phospholipase C	4.25
lmo0202	*hly*	Listeriolysin O precursor	4.23
lmo0203	*mpl*	Zinc metalloproteinase precursor	3.37
lmo0204	*actA*	Actin-assembly inducing protein precursor	7.74
lmo0205	*plcB*	Phospholipase C	8.02
lmo0206	*orfX*	Conserved hypothetical protein	4.12
lmo0838	*uhpT*	Sugar phosphate antiporter	4.60

*$LO28\_t_0/LO28\_t_{60}$. The complete microarray datasets can be found in Supplementary Tables S2 and S3 and in the GEO database (http://www.ncbi.nlm.nih.gov/geo/), GEO Series GSE32913.

allowing a direct comparison of all data obtained. Notably, the growth of the wild-type, Δ*lisR* and Δ*cesR* mutant strains were similar within the time-frame of this experiment (Supplementary Fig. S1). The microarray data were validated by qRT-PCR analysis of 30 genes (Supplementary Fig. S2 and Supplementary Tables S2 and S3).

When focusing on the 73 genes induced at least fourfold by cefuroxime exposure in the wild-type strain, we observed that 26 genes (36 %) were activated by LisR or CesR (see Table 2). In addition, four VirRS- and nine LiaSR-regulated genes were found among the genes induced at least fourfold by cefuroxime exposure (Table 2). Thus, 53 % of the genes highly upregulated in response to cefuroxime exposure are under positive control by one of the four TCSs. Strikingly, LisR, CesR and LiaSR together activate the nine genes induced at least 10-fold by cefuroxime exposure in the wild-type strain (Table 2). Collectively, these results highlight the importance of two-component signal transduction systems in orchestrating the response to cell envelope stress in *L. monocytogenes*.

The LisR- and CesR-mediated regulation observed by transcriptome profiling is most likely to be the result of both primary (direct) and secondary (indirect) effects, resulting from regulation of primary targets. The LisR-binding sequence is currently unknown, so in order to identify genes under the direct control of LisR, we searched for conserved sequences in the putative promoter regions ranging from position −1 to −400 relative to the translation start sites of the LisR-regulated genes (see Methods for details). Unfortunately, our search for conserved LisR binding boxes upstream from the 31 LisR-regulated genes was unsuccessful. In a previous study, we identified a conserved CesR-binding sequence (AATCTTTAA) in the promoter regions of four CesR-regulated genes (Gottschalk *et al.*, 2008). When searching among the 19 genes induced by cefuroxime exposure in a CesR-dependent manner, putative CesR-binding boxes were found upstream of 14 genes (see Supplementary Fig. S3), suggesting that these genes are under direct control of CesRK.

Characterization of selected genes belonging to the cefuroxime stimulon with respect to tolerance to cell-envelope-perturbing conditions

To investigate if genes belonging to the cefuroxime stimulon are induced in response to cell-envelope-perturbing conditions encountered by *L. monocytogenes in vitro* and/or *in vivo*, we studied the expression of nine selected genes (*htrA*, *lmo1037*, *lmo1416*, *lmo1518*, *lmo2210*, *lmo2442*, *lmo2522*, *lmo2568* and *lmo2812*) in response to ampicillin, ethanol, bile salts and sodium chloride. Besides being highly upregulated in response to cefuroxime exposure, eight of the selected genes also showed differential regulation by LisR or CesR, whereas a single gene is controlled by LiaR (Table 2) (Fritsch *et al.*, 2011). The relative expression of these nine selected genes in cells harvested prior to addition of selected stress agents was compared with that of cells harvested after 1 hour growth in the presence of subinhibitory concentrations of the stress agent. As a control for growth-related effects, expression ratios comparing time zero with a non-stressed sample harvested at time 60 min were included. In the wild-type strain, all nine genes were induced by the five agents tested (Fig. 1). Importantly, no growth-related effects were observed (Fig. 1, control). The expression of the genes designated as being part of the CesRK-regulon (*lmo1037*, *lmo1416*, *lmo2442* and *lmo2812)* were highly stimulated by the addition of ethanol and sodium chloride, whereas no specific pattern of induction was observed for the LisR-regulated genes (*htrA*, *lmo1518*, *lmo2210* and *lmo2522*) and the LiaR-regulated gene (*lmo2568*). qRT-PCR experiments performed in *lisR* and *cesR* mutant backgrounds showed that that full induction of the LisR- and CesR-regulated genes depended on the presence of their corresponding regulator, whereas induction of *lmo2568* by all five agents tested was unaffected by the absence of *lisR* or *cesR* (data not shown).

Of the nine genes analysed by qRT-PCR, only two genes (*htrA* and *lmo2812*) encode proteins with known functions: HtrA and PBPD2. To investigate the roles of *lmo1037*, *lmo1518*, *lmo2442*, *lmo2522* and *lmo2568* during growth under cell-envelope-perturbing conditions, we constructed mutant strains carrying in-frame deletions in these five genes. In our previous study, mutants carrying in-frame deletions of *lmo1416*, *lmo2210* and *lmo2812* were constructed and analysed with respect to ethanol tolerance and antibiotic resistance using disk diffusion assays (Gottschalk *et al.*, 2008). These mutants were also included in the present study to obtain more information on their roles in the cell-envelope-stress response. Furthermore, the wild-type, Δ*lisR* and Δ*cesR* mutant strains were included as controls. The 11 strains were analysed with respect to growth in the presence of 4 μg cefuroxime ml^{-1}, 8 % sodium chloride, 0.08 % bile salts, 0.1 % SDS and 5 % ethanol.

Under standard growth conditions (BHI medium, 37 °C), no differences in growth were observed between the wild-type strain and ten deletion mutants (data not shown). When overnight cultures were diluted into BHI medium containing 4 μg cefuroxime ml^{-1}, growth of Δ*lisR*, Δ*cesR* and Δ*lmo1416* was inhibited (Fig. 2a). Of the residual seven mutants tested, only a single mutant (Δ*lmo2522*) was impaired in comparison with the wild-type strain (Fig. 2a). In the presence of 8 % sodium chloride, the growth of Δ*lmo2522* and Δ*cesR* was found to be impaired, whereas Δ*lisR* and the other mutants tested displayed growth similar to the wild-type, although LisR has previously been linked to osmotolerance (Fig. 2b) (Sleator & Hill, 2005). BHI medium containing 0.08 % bile salts prevented growth of the Δ*lisR* strain, whereas growth of Δ*cesR*, Δ*lmo1416*, Δ*lmo2210* and Δ*lmo2812* was impaired in comparison with the wild-type strain (Fig. 2c), and a similar pattern was observed when testing growth in the presence of 0.1 % SDS (data not shown). The Δ*cesR*, Δ*lisR* and Δ*lmo1416* mutant strains have previously been shown to be tolerant to ethanol (Gottschalk *et al.*, 2008). In contrast, the wild-type and the seven other mutant strains under investigation were unable to grow in

Table 2. Differentially expressed genes (at least fourfold change) in *L. monocytogenes* LO28 wild-type, Δ*lisR* and Δ*cesR* mutant strains after 1 h of growth in the presence of 4 μg cefuroxime ml^{-1}, in comparison with expression at time zero

Genes were clustered into four groups on the basis of the TCSs controlling their expression: LisRK (this study), CesRK (this study), LiaSR (Fritsch *et al.*, 2011) and VirRS (Mandin *et al.*, 2005).

Gene	Name	Genome organization*	Protein description	Microarray ratio†		
				LO28	Δ*lisR*	Δ*cesR*
LisR regulated genes						
lmo0211	*ctc*	Monocistronic	50S ribosomal protein L25/general stress protein Ctc	5.46	2.46	5.35
lmo0292	*htrA*	op 52 (*lmo0291–0292*)	Similar to heat-shock protein htrA serine protease	18.39	1.20	21.14
lmo0802	–	Monocistronic	Weakly similar to GTP-pyrophosphokinase	5.1	0.72	4.08
lmo1315	–	op 209 (*lmo1315–1327*)	Similar to undecaprenyl diphosphate synthase	4.62	0.88	3.79
lmo1377	*lisR*	op 220 (*lmo1377–1378*)	Two-component response regulator	4.17	1.06	3.86
lmo1518‡	–	Monocistronic	Conserved hypothetical protein.	6.91	2.24	10.80
lmo1680	–	op 290 (*lmo1681–1678*)	Similar to cystathionine gamma-synthase	9.53	4.45	7.11
lmo1681	–	op 290	Similar to cobalamin-independent methionine synthase	7.33	3.09	4.16
lmo1690	–	Monocistronic	Similar to membrane-bound metal-dependent hydrolase	12.48	1.88	13.00
lmo1698	–	Monocistronic	Similar to ribosomal-protein-alanine *N*-acetyltransferase	4.19	2.07	3.38
lmo1919	–	Monocistronic	Zn-dependent protease	4.81	1.09	4.96
lmo2210§	–	Monocistronic	Conserved hypothetical protein	13.8	0.86	30.67
lmo2350	–	op 419 (*lmo2351–2343*)	Conserved hypothetical protein	5.76	2.80	5.94
lmo2522‡	–	Monocistronic	Similar to cell-wall-binding protein	34.21	8.98	18.59
lmo2720	–	Monocistronic	Similar to acetate-CoA ligase	5.89	2.79	3.24
CesR regulated genes						
lmo0443	–	Monocistronic	Similar to transcription regulator of the LytR family	4.87	7.06	1.57
lmo1037‡	–	Monocistronic	Integral membrane protein YoaT homologue	10.8	16.36	1.17
lmo1215	–	Monocistronic	Similar to *N*-acetylmuramoyl-L-alanine amidase (autolysin)	4.39	4.43	1.89
lmo1416§	–	op 231 (*lmo1420–1416*)	Similar to glycopeptide antibiotics resistance protein	19.95	39.12	6.65
lmo2120	*dacA*	op 388 (*lmo2120–2119*)	Similar to di-adenylate cyclase	4.37	4.60	1.66
lmo2420	–	op 432 (*lmo2422–2420*)	Conserved hypothetical protein	4.02	8.93	0.91
lmo2442‡	–	Monocistronic	Conserved hypothetical protein	14.37	26.13	1.46
lmo2687	–	op 483 (*2689–2686*)	Similar to cell division protein FtsW	4.19	7.35	1.32
lmo2688	–	op 483	Similar to cell division protein FtsW	4.12	8.08	1.24
lmo2689	–	op 483	Highly similar to Mg^{2+} transport ATPase	4.89	9.78	2.43
lmo2812§	*pbpD2*	Monocistronic	Similar to D-alanyl-D-alanine carboxypeptidase	12.12	50.50	1.26
LiaSR regulated genes						
lmo0954	*liaI*	op 149 (*lmo0954–0955*)	Conserved hypothetical protein	6.79	4.44	6.79
lmo0955	*liaH*	op 149	Phage-shock protein A homologue	5.96	5.42	7.45
lmo1966	–	op 356 (*lmo1966–1967*)	Conserved hypothetical protein	4.99	3.84	4.84
lmo2484	–	op 441 (*lmo2487–2484*)	*B. subtilis* YvlD homologue	4.99	6.48	4.89
lmo2485	–	op 441	Similar to stress-responsive transcription regulator	5.06	6.10	6.66
lmo2486	–	op 441	Conserved hypothetical protein	6.15	6.68	7.15
lmo2487	–	op 441	*B. subtilis* YvlB homologue	5.78	8.03	7.92
lmo2567	–	Monocistronic	Conserved hypothetical protein	10.97	15.24	11.92
lmo2568‡	–	Monocistronic	Conserved hypothetical protein	9.18	14.57	12.75
VirRS regulated genes						
lmo0972	*dltC*	op 155 (*lmo0974–0971*)	D-alanine-poly(phosphoribitol) ligase subunit 2	4.82	6.26	5.18
lmo0973	*dltB*	op 155	D-alanyl transfer protein (teichoic acid)	4.96	7.52	5.51
lmo0974	*dltA*	op 155	D-alanine-D-alanyl carrier protein ligase	6.53	7.68	6.22
lmo2177	–	Monocistronic	Conserved hypothetical protein	5.08	5.18	4.00

*According to Toledo-Arana *et al.* (2009).

†LO28_t_0/LO28_t_{60}. The complete microarray datasets can be found in Supplementary Tables S2 and S3 and in the GEO database (http://www.ncbi.nlm.nih.gov/geo/), GEO Series GSE32913.

‡Genes selected for construction of in-frame deletion mutants in the present study.

§In-frame deletion strain constructed by Gottschalk *et al.* (2008).

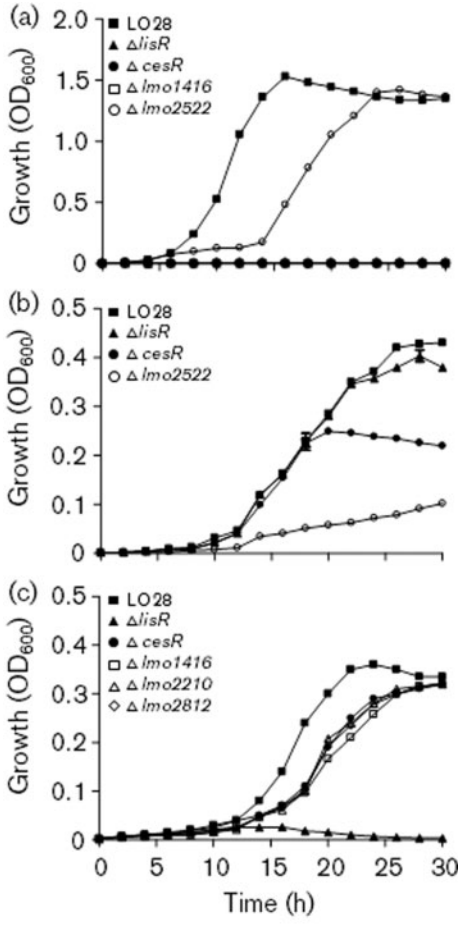

Fig. 2. Stress tolerance assay. Overnight cultures of wild-type and mutant strains were diluted into BHI medium supplemented with (a) 4 μg cefuroxime ml^{-1}, (b) 8 % sodium chloride or (c) 0.08 % bile salt. The growth of wild-type and mutant strains carrying in-frame deletions of *lisR*, *cesR*, *lmo1037*, *lmo1416*, *lmo1518*, *lmo2210*, *lmo2442*, *lmo2522*, *lmo2568* and *lmo2812* was monitored. Data for strains exhibiting growth defects compared with the wild-type strain are shown. Error bars represent the SDs of three independent experiments. Data for the presented deletion strains were significantly different from the wild-type ($P<0.05$) for at least four data points.

the presence of 5 % ethanol (data not shown). Collectively, the growth experiments demonstrated novel roles for *lisR*, *cesR*, *lmo1416*, *lmo2210* and *lmo2812* in the tolerance of *L. monocytogenes* to bile salts and SDS, whereas *lmo2522* was shown to contribute to growth in the presence of cefuroxime and sodium chloride.

MICs for cefuroxime, ampicillin and gentamicin in wild-type *L. monocytogenes* and deletion mutant strains

Patients suffering from listeriosis are typically treated with ampicillin and the aminoglycoside gentamicin, whereas the cephalosporins (including cefuroxime) are generally inactive against listeria (Hof, 2003; Temple & Nahata, 2000). To investigate the response of the mutant strains to these antibiotics, we determined the MIC for cefuroxime, ampicillin and gentamicin, by performing an E-test on Mueller–Hinton agar plates with 5 % sheep blood (Table 3). For Δ*lisR*, Δ*cesR* and Δ*lmo1416* a four- to fivefold increased sensitivity to cefuroxime was observed relative to that of the wild-type, whereas a small (twofold or less) but significant increase in sensitivity was observed for Δ*lmo2210*, Δ*lmo2442*, Δ*lmo2522* and Δ*lmo2568*. Only the Δ*cesR* and Δ*lmo1416* mutants were found to be significantly more sensitive to ampicillin relative to the wild-type (twofold decrease in MIC). Finally, the sensitivity of all ten mutant strains to gentamicin was similar to that of the wild-type strain (Table 3). These results suggest that several CesR- and LisR-regulated genes play a role in the protection of *L. monocytogenes* against cefuroxime.

DISCUSSION

In *L. monocytogenes*, the global response to agents affecting the integrity of the cell envelope is not well understood. Transcriptome analyses have been performed for studies on *L. monocytogenes* subjected to general stresses, such as heat-shock, hypoxia and low temperature (Toledo-Arana *et al.*, 2009; van der Veen *et al.*, 2007). However, microarray analyses of the response to cell-envelope-active agents, such as cell-wall-targeting antibiotics, are lacking. In contrast to this, the cell envelope stress response has been studied very

Table 3. Determination of the MIC (μg ml^{-1}) for wild-type LO28 and in-frame deletion mutants

The experiments were performed in triplicate with identical results. Values indicating at least twofold sensitivity of the mutant relative to the wild-type strain are shown in bold type.

	LO28	*lisR*	*cesR*	*lmo1037**	*lmo1518**	*lmo1416*†	*lmo2210*†	*lmo2442**	*lmo2522**	*lmo2568**	*lmo2812*†
Cefuroxime	4	**1**	**1**	3	3	**0.75**	**2**	**2**	2.5	**2**	3
Ampicillin	0.094	0.079	**0.047**	0.094	0.094	**0.047**	0.094	0.094	0.094	0.094	0.157
Gentamicin	0.125	0.125	0.125	0.125	0.125	0.157	0.157	0.125	0.157	0.125	0.157

*In-frame deletion mutants constructed in the present study.
†In-frame deletion strains constructed by Gottschalk *et al.* (2008).

intensively in the Gram-positive model organism *Bacillus subtilis*. Transcriptome analyses have revealed a complex network comprising multiple extracytoplasmic function (ECF) σ factors and TCSs orchestrating the response of *B. subtilis* to cell-envelope-active compounds such as vancomycin, bacitracin and CAMPs (Cao *et al.*, 2002; Mascher *et al.*, 2003; Pietiäinen *et al.*, 2005; Wecke & Mascher, 2011). Likewise, transcriptional profiling of the response of the Gram-positive human pathogen *Staphylococcus aureus* to antibiotics such as vancomycin, oxacillin, bacitracin and D-cycloserine have been performed, which showed that the LiaSR-like TCS VraSR plays a key role in the *S. aureus* cell envelope stress response (Kuroda *et al.*, 2003; Utaida *et al.*, 2003; Wecke & Mascher, 2011).

In this study, we investigated the cell envelope stress response of *L. monocytogenes* induced by the β-lactam antibiotic cefuroxime. The majority of the genes highly upregulated by cefuroxime exposure are predicted to have cell-envelope-related functions, including the highly conserved 'marker' loci of the Gram-positive cell envelope stress response: the Dlt system and the alternative PBPs, serve to maintain the integrity of the bacterial cell envelope (Jordan *et al.*, 2008). Because cefuroxime acts by inhibiting the enzymic functions of the PBPs, the upregulation of five out of nine PBPs by *L. monocytogenes* can be viewed as an attempt to compensate for the damaging effects of this cell-wall-acting antibiotic. The *dlt*-operon in *L. monocytogenes* is known to be controlled by VirRS, whereas upregulation of the PBPD2-encoding gene *lmo2812* is mediated by CesRK. Interestingly, 53 % of the genes upregulated at least fourfold by cefuroxime were found to be under control by one of the four TCSs linked to cell envelope stress response in *L. monocytogenes*: LisRK, CesRK, LiaSR and VirRS. The LiaSR and VirRS regulons did not overlap with the genes regulated by LisRK and CesRK; however, several LisR-activated genes were negatively affected by CesR and vice versa, suggesting a link between these two TCSs. In contrast, no link between LisRK and LiaSR was observed, although this has been suggested by others (Cotter *et al.*, 2002). Several highly upregulated genes could not be connected to any of the four TCSs, suggesting that additional gene regulatory systems must participate in mediating their upregulation in response to cefuroxime exposure. In *B. subtilis*, the cell envelope stress response involves at least three out of seven ECF σ factors (i.e. σ^M, σ^W and σ^X), whereas in *S. aureus*, ECF σ-factors are absent (Jordan *et al.*, 2008). *L. monocytogenes* contains a single ECF σ factor named σ^C (*lmo0423*), which has been shown to contribute to heat tolerance; however, no apparent link to the cell wall stress response has been observed (Zhang *et al.*, 2005). The general stress sigma factor σ^B contributes to the tolerance of *L. monocytogenes* to cell-wall-acting antibiotics (Begley *et al.*, 2006). However, only a few members of the σ^B-regulon were observed among the genes highly induced by cefuroxime exposure (Supplementary Table S4). In addition to multiple ECF σ-factors, the cell-envelope-stress response in *B. subtilis* involves at least four TCS systems (BceRS, LiaSR, YvcPQ and YxdJK) with homology to LiaSR or VirRS (Jordan *et al.*, 2008). *S. aureus* also relies on multiple TCSs to coordinate the response to cell wall antibiotics and CAMPs, such as the LiaSR-like system VraSR and the VirRS-like system GraSR (Falord *et al.*, 2011; Kuroda *et al.*, 2003; Pietiäinen *et al.*, 2009). Curiously, CesRK-like systems are absent in both *S. aureus* and *B. subtilis*, whereas in *Enterococcus faecalis*, the CesRK-like system CroRS has been shown to play a role in the intrinsic resistance to β-lactam antibiotics (Comenge *et al.*, 2003; Hancock & Perego, 2004). In *S. aureus*, a LisRK-like system, ArlRS, has been shown to be involved in the control of autolysis, cell growth and virulence (Fournier *et al.*, 2001; Fournier & Hooper, 2000; Liang *et al.*, 2005); however in *B. subtilis*, LisRK-like systems are absent (Jordan *et al.*, 2008). Collectively, these observations suggest that in *L. monocytogenes*, a regulatory network consisting of at least four TCSs is orchestrating the response to cell envelope stress. The extent of the interplay between CesRK, LisRK, LiaSR and VirRS, as well as the involvement of additional regulators in the cell-envelope-stress response, may be revealed by performing comparative global transcriptome analyses of the response to other cell-envelope-acting agents.

In addition to cefuroxime, other agents known to affect cell envelope integrity were capable of inducing the expression of genes controlled by LisRK, CesRK or LiaSR. Several of these highly upregulated genes were found to contribute to the innate resistance of *L. monocytogenes* to cefuroxime, and a subset of these contributes to the tolerance of *L. monocytogenes* against bile salts, SDS, ethanol and sodium chloride. The finding that sodium chloride stimulates the expression of all nine genes tested suggests that the regulatory systems coordinating the cell-envelope-stress response in *L. monocytogenes* are linked to the osmotic stress response. Interestingly, we observed that the highly upregulated gene *lmo2522* plays an important role in *L. monocytogenes* tolerance to osmotic stress; however, the function of the cell-wall-associated LMO2522 protein remains to be elucidated.

L. monocytogenes is capable of causing systemic infections in humans, leading to serious conditions such as meningitis or encephalitis, and in the case of pregnant women, abortion or stillbirth (Vázquez-Boland *et al.*, 2001). After ingestion of contaminated foods, *L. monocytogenes* multiplies within the gut. In susceptible humans, the bacterium may cross the intestinal epithelium and enter the bloodstream. It is capable of multiplying in the liver and spleen, and it may re-enter the bloodstream to cause systemic or central nervous system infections, or fetal infections in pregnant women. In recent years, several studies have aimed at determining the global transcriptional profile of *L. monocytogenes* at various stages of infection, including *L. monocytogenes* residing in the gastrointestinal tract, in blood and during intracellular infection (Camejo *et al.*, 2009; Chatterjee *et al.*, 2006; Joseph *et al.*, 2006; Toledo-Arana *et al.*, 2009). Interestingly, the majority of genes highly upregulated upon cefuroxime

exposure are also induced when *L. monocytogenes* resides in blood and during intracellular infection of mammalian cells. Furthermore, among the downregulated genes, we observed a large overlap with the transcriptome of *L. monocytogenes* exposed to mammalian bile, including a significant repression of genes belonging to the PrfA virulence regulon required for invasion and replication in mammalian cells. Thus, the cell envelope stress response of *L. monocytogenes* appears to be activated at multiple stages of the infectious process. In accordance with this, LisRK, CesRK and VirRS have been shown to contribute to pathogenesis in mice, and several of the genes shown to be highly upregulated in response to cefuroxime exposure play important roles in virulence, including *dacA*, *lmo2714*, *htrA* and the *dlt*-operon (Abachin *et al.*, 2002; Camejo *et al.*, 2009; Stack *et al.*, 2005; Wilson *et al.*, 2006; Woodward *et al.*, 2010).

In summary, this study demonstrated central roles for LisRK and CesRK, in addition to VirRS and LiaSR, in the cell envelope stress response of *L. monocytogenes*. Furthermore, we showed that several genes highly induced in response to cefuroxime exposure contribute to the tolerance of *L. monocytogenes* to cell-envelope-perturbing conditions. These results suggest an important role for the cell envelope stress response in the adaptation of *L. monocytogenes* to a variety of *in vitro* and *in vivo* environments.

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