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σ^B-dependent expression patterns of compatible solute transporter genes *opuCA* and *lmo1421* and the conjugated bile salt hydrolase gene *bsh* in *Listeria monocytogenes*

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Listeria monocytogenes is a food-borne pathogen that can persist and grow under a wide variety of environmental conditions including low pH and high osmolarity. The alternative sigma factor σ^B contributes to *L. monocytogenes* survival under extreme conditions. The purpose of this study was to identify and confirm specific σ^B-dependent genes in *L. monocytogenes* and to characterize their expression patterns under various stress conditions. *opuCA*, *lmo1421* and *bsh* were identified as putative σ^B-dependent genes based on the presence of a predicted σ^B-dependent promoter sequence upstream of each gene. *opuCA* and *lmo1421* encode known and putative compatible solute transporter proteins, respectively, and *bsh* encodes a conjugated bile salt hydrolase (BSH). Reporter fusions and semi-quantitative RT-PCR techniques were used to confirm σ^B-dependent regulation of these stress-response genes and to determine their expression patterns in response to environmental stresses. RT-PCR demonstrated that *opuCA*, *lmo1421* and *bsh* transcript levels are reduced in stationary-phase *L. monocytogenes* Δ*sigB* cells relative to levels present in wild-type cells. Furthermore, BSH activity is abolished in a *L. monocytogenes* Δ*sigB* strain. RT-PCR confirmed growth-phase-dependent expression of *opuCA*, with highest levels of expression in stationary-phase cells. The *L. monocytogenes* wild-type strain exhibited two- and threefold induction of *opuCA* expression and seven- and fivefold induction of *lmo1421* expression following 10 and 15 min exposure to 0·5 M KCl, respectively, as determined by RT-PCR, suggesting rapid induction of σ^B activity in exponential-phase *L. monocytogenes* upon exposure to salt stress. Single-copy chromosomal *opuCA–gus* reporter fusions also showed significant induction of *opuCA* expression following exposure of exponential-phase cells to increased salt concentrations (0·5 M NaCl or 0·5 M KCl). In conjunction with recent findings that indicate a role for *opuCA* and *bsh* in *L. monocytogenes* virulence, the data presented here provide further evidence of specific σ^B-mediated contributions to both environmental stress resistance and intra-host survival in *L. monocytogenes*.

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INTRODUCTION

Nearly 28 % of all deaths caused by known food-borne pathogens in the USA are attributed to infections with the Gram-positive bacterium *Listeria monocytogenes* (Mead *et al.*, 1999). Symptoms of systemic listeriosis include septicaemia, meningitis and spontaneous abortion. Individuals with compromised immune systems, neonates and the elderly are at the highest risk of contracting listeriosis (Farber & Peterkin, 1991). *L. monocytogenes* is considered ubiquitous in nature. This organism survives and multiplies under a wide spectrum of environmental conditions, including in food-processing plants and in a variety of ready-to-eat food products. Growth of *L. monocytogenes* has been documented at temperatures ranging from −0·4 to +45 °C (Farber & Peterkin, 1991) and survival has been observed under acidic conditions (e.g. pH 2·5) and in solutions of high (10–20 % NaCl) osmolarity (Cole *et al.*, 1990; Sleator *et al.*, 2000). *L. monocytogenes* may encounter refrigeration temperatures, low pH and high osmolarity at various stages of food processing (Farber & Peterkin, 1991; Lou & Yousef, 1999). Following consumption of a contaminated food product by a potential host, *L. monocytogenes* encounters additional stressful environmental conditions during gastrointestinal passage, including exposure to proteolytic enzymes, the stomach's acidic environment (as low as pH 2·0), bile salts (Davenport, 1982) and an

Abbreviations: BSH, bile salt hydrolase; GUS, β-glucuronidase; MU⁻, methylumbelliferone.

intestinal osmolarity equivalent to 0·3 M NaCl (Chowdhury *et al.*, 1996).

Bacteria can alter gene expression patterns at appropriate times in response to changing environments and stressful conditions. Both Gram-positive and Gram-negative bacteria have the ability to regulate patterns of gene expression at the transcriptional level (Becker *et al.*, 2000; Fang *et al.*, 1992; Helmann *et al.*, 2001). The holoenzyme RNA polymerase (RNAP) catalyses the transcription of DNA into mRNA (Burgess *et al.*, 1969). A sigma factor is a protein subunit of RNAP that is required for recognition of specific promoter sequences and for initiation of transcription (Helmann & Chamberlin, 1988). The association of different alternative sigma factors with RNAP is one mechanism that enables a bacterial cell to rapidly induce expression of specific genes within a regulon in response to specific stimuli. The general stress-responsive alternative sigma factor σ^S has been identified in many Gram-negative bacteria, including *Escherichia coli*, *Salmonella* spp. and *Yersinia* spp. (Badger & Miller, 1995; Fang *et al.*, 1992; McCann *et al.*, 1991). In both *E. coli* and *Salmonella* Typhimurium, σ^S plays a crucial role in protection against conditions of starvation, hyperosmolarity, oxidative and acid stresses (Cheville *et al.*, 1996; Small *et al.*, 1994). For Gram-positive bacteria, the general stress-responsive alternative sigma factor σ^B was first identified and characterized in *Bacillus subtilis* (Boylan *et al.*, 1993). The σ^B-dependent general stress regulon of *B. subtilis* consists of well over 100 genes that are induced by exposure to stressful conditions such as heat, acid, ethanol or high osmolarity, or by deprivation of glucose, oxygen or phosphate (Helmann *et al.*, 2001; Petersohn *et al.*, 2001; Price *et al.*, 2001). Previous studies have demonstrated roles for stress-responsive sigma factors in regulating expression of virulence genes in some bacterial pathogens, including *Staphylococcus aureus*, *Yersinia enterocolitica* and *Salmonella* (Deora *et al.*, 1997; Humphreys *et al.*, 1999; Kullik *et al.*, 1998), suggesting a link between stress response and virulence in these organisms.

Mounting evidence also supports an association between the ability of *L. monocytogenes* to survive exposure to environmental stresses and to infect host cells. For example, σ^B contributes to *L. monocytogenes* survival and growth under certain environmental stress conditions [e.g. acid stress, low-temperature stress, salt stress (Becker *et al.*, 1998, 2000)], as well as to persistence within a host and to host cell infection (Nadon *et al.*, 2002; Wiedmann *et al.*, 1998). The stress-responsive compatible solute transporter *opuCA* has also been demonstrated to contribute to host infection in an animal model (Sleator *et al.*, 2001). While transcription of selected *L. monocytogenes* genes (e.g. *opuCA*, *lmo1421*) has been shown to be reduced in a *sigB* null mutant background (Ferreira *et al.*, 2003; Fraser *et al.*, 2003), the temporal nature of σ^B-dependent contributions to transcription induction of these genes has not yet been quantified. To test our hypothesis that σ^B coordinates a rapid response that aids *L. monocytogenes* in survival of environmental and host-imposed stress conditions, we identified and confirmed the σ^B dependence of *L. monocytogenes* genes that had previously been demonstrated to contribute to survival under these conditions and characterized their induction and expression patterns. Specifically, we confirmed the σ^B dependence of *L. monocytogenes opuCA*, *lmo1421* and *bsh*. These genes represent general stress-response genes (*opuCA* and *lmo1421* encode known and putative compatible solute transporter proteins, respectively) and a virulence gene [*bsh* encodes a conjugated bile salt hydrolase (BSH)]. We have shown that all three genes are expressed under conditions of environmental stress and that expression of *opuCA* and *lmo1421* is induced following exposure to salt stress.

METHODS

Bacterial strains. *L. monocytogenes* strains used in this study are described in Table 1. *Listeria innocua* wild-type strain DD680 was used in the BSH assay only.

Growth conditions and cell collection. To characterize σ^B-dependent expression of the target genes in stationary-phase cells, a *L. monocytogenes* wild-type strain (10403S) and a *sigB* null mutant (Δ*sigB*) were grown for 12 h (30 °C, shaking at 250 r.p.m.) in Brain–Heart Infusion Broth (BHI). Stationary-phase cells grown in this manner were harvested and used for total RNA isolation and subsequent RT-PCR experiments as described below. To monitor target gene expression throughout growth, overnight cultures of the *L. monocytogenes* wild-type and Δ*sigB* strains grown to stationary phase as described above were diluted 1 : 1000 into 10 ml of BHI broth and incubated at 30 °C (shaking at 250 r.p.m.). When these cultures reached an OD_{600} value of 0·4, they were diluted again (1 : 200) into a side-arm flask containing 150 ml of BHI and incubated at 30 °C with shaking at 250 r.p.m. When these cultures reached an OD_{600} value of 0·4, 0·8 or 0·8 + 1 h, 20 ml of broth were centrifuged (8800 ***g***) and the harvested cells were used for total RNA isolation and RT-PCR experiments.

For detection of BSH activity, the *L. monocytogenes* wild-type,

Table 1. Bacterial strains used in this study

Strain	Characteristics	Reference
10403S	*L. monocytogenes* wild-type strain	Portnoy *et al.* (1988)
FSL A1-254	*L. monocytogenes sigB* in-frame deletion	Wiedmann *et al.* (1998)
FSL S1-063	*L. monocytogenes opuCA–gus* fusion, *sigB* wild-type	Ferreira *et al.* (2003)
FSL S1-059	*L. monocytogenes opuCA–gus* fusion, Δ*sigB*	Ferreira *et al.* (2003)
L. innocua DD680	*L. innocua* wild-type strain	Lab. strain collection

L. innocua wild-type (negative control) and Δ*sigB* null mutant strains were spotted onto deMan, Rogosa and Sharpe (MRS) agar medium (BD Biosciences) containing 0·5 % (w/v) glycodeoxycholic acid (Sigma) as originally described by Dashkevicz & Feigner (Dashkevicz & Feighner, 1989; Dussurget *et al.*, 2002).

Salt-stress conditions. To monitor induction of target gene expression following exposure to salt stress, bacterial strains were grown to mid-exponential phase in BHI as described above. Specifically, 20 ml aliquots of cells grown to an OD_{600} value of 0·4 were centrifuged as described above, then each pellet was resuspended in 20 ml of 0·154 M NaCl (representing a physiological salt concentration of 0·9 %, w/v; pH 5·9), 0·5 M NaCl (pH 5·8) or 0·5 M KCl (pH 5·8). Following exposure times of 15, 30, 60 or 120 min, 1 ml samples of the wild-type and Δ*sigB opuCA–gus* fusion strains (FSL S1-063 and FSL S1-059, respectively) were collected for β-glucuronidase (GUS) activity measurement, as described below.

L. monocytogenes wild-type and Δ*sigB* strains (10403S and FSL A1-254, respectively) were also exposed to 0·121 M KCl (0·9 %) and 0·5 M KCl as described above. Samples were collected at 5, 10 and 15 min post-exposure for total RNA isolation and RT-PCR.

Total RNA isolation. For the RT-PCR experiments, total RNA was purified from cells collected during exposure to salt stress and throughout growth, as described above. Bacterial cells collected at the specified time points were centrifuged and immediately resuspended in 10 ml Trizol reagent (Invitrogen) per 30 ml of culture harvested. The resuspension was immediately placed on ice and sonicated for three 20 s intervals (output: 20 W) using a Sonicator 3000 (Misonix). A 20 ml aliquot of chloroform (Shelton Scientific) was added for each 10 ml of original cell culture. After vigorous vortexing and 10 min incubation at room temperature, tubes were centrifuged (2190 ***g***) for 60 min. Nucleic acids from the aqueous layer were precipitated with an equal volume of 2-propanol and centrifuged (17 900 ***g***). The resulting pellets were washed twice with 100 % ethanol, resuspended in RQ1 10× DNase Buffer and treated with RQ1 DNase (Promega). Nucleic acids were subsequently purified by phenol/chloroform extraction and ethanol precipitation with 0·3 M sodium acetate (Sambrook *et al.*, 1989). DNase treatment, phenol/chloroform extraction and ethanol precipitation steps were repeated two additional times to remove any contaminating DNA. The final RNA pellet was resuspended in 60 µl diethyl pyrocarbonate (DEPC)-treated water (Invitrogen). Total nucleic acid concentrations were estimated using absorbance readings (260 nm/280 nm) on a DU Series 600 Spectrophotometer (Beckman Coulter).

RT-PCR. Reverse transcription was performed using the Superscript First-Strand Synthesis RT-PCR System (Invitrogen) with 50 ng of total RNA for each reaction. Primers to amplify *opuCA*, *lmo1421*, *bsh* and *rpoB*, which were designed using PRIMEREXPRESS software (Applied Biosystems), are shown in Table 2. Reverse transcription reactions were cycled once at 42 °C for 50 min and then at 70 °C for 15 min. PCR amplification of cDNA was performed using 10 µl of each reverse transcriptase reaction and the Ampli*Taq* Gold DNA Polymerase system (Applied Biosystems). PCR cycling conditions included an initial 9 min hold at 95 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C, 30 s at 72 °C, and a final hold of 5 min at 72 °C. Reverse transcription and PCR amplification reactions were performed in a GeneAmp 9600 (Perkin Elmer). To monitor for possible contamination by genomic DNA, an aliquot of each RT-PCR was run in the absence of Superscript II enzyme. RT-PCR products (10 µl) were subjected to gel electrophoresis using 3 % Metaphor (BioWhittaker Molecular Applications) agarose gels. The pGEM DNA ladder was used as a molecular mass marker and as a standard for PCR product quantification.

For quantification of the RT-PCR products, LABIMAGE software (Kapelan) was used to analyse gel images following ethidium bromide staining. All RT-PCR product bands were compared against pGEM ladder bands of known size and concentration. The constitutively expressed *rpoB*, which is not σ^B-dependent in *L. monocytogenes*, served as an internal control gene for these RT-PCR experiments (see Results). To account for variation between RT-PCRs and RNA collections, the DNA quantities from *lmo1421*, *opuCA* and *bsh* RT-PCR were each normalized to the quantities of *rpoB* RT-PCR products that were generated in each experiment. For RT-PCR analysis of *opuCA* throughout growth, *opuCA* transcripts were quantified as relative *opuCA* product quantities normalized to *rpoB* quantities at corresponding time-collection points. For the salt-stress induction experiments, DNA quantities for *lmo1421*, *opuCA* and *bsh* RT-PCR products were also normalized to *rpoB* RT-PCR product quantities to give relative RT-PCR product quantities. To measure induction, the normalized RT-PCR product quantities from cells exposed to salt stress (0·5 M KCl) were divided by the corresponding normalized RT-PCR product quantities for non-exposed cells (0·121 M KCl).

Quantitative GUS assay. Assays for GUS activity were performed essentially as described by Youngman (Harwood & Cutting, 1990). Specifically, cells from 1 ml aliquots were pelleted and washed in 1 ml Buffer AB Light (60 mM K_2HPO_4, 40 mM KH_2PO_4, 0·1 M NaCl, pH 7·0). The washed cell pellet was resuspended in 400 µl of Buffer AB Light for GUS assay and standard plate counts on BHI agar. To quantify GUS activities, 100 µl of the cell suspension were thoroughly mixed with 100 µl of Buffer AB Plus (Buffer AB Light containing 0·2 % Triton X-100) and incubated at room temperature for 60 min to lyse the cells. In a black, flat-bottomed Packard OptiPlate 96-well plate (Perkin Elmer), 50 µl of the lysed cells were mixed with 10 µl of 4-methylumbelliferyl-β-D-glucuronide (4-MUG; Sigma) in 0·4 mg ml^{-1} DMSO (Fisher Scientific) and held at room temperature for at least 60 min. Exact incubation times were recorded

Table 2. Primers used for RT-PCR analyses

Gene	Primer name	Primer sequence
opuCA	TqMnOpuF1	5′-ACA TCG ATA AAG GAG AAT TTG TTT GTT-3′
	TqMnOpuR1	5′-GCC GGT TAA TCA TCT TCA TTG TT-3′
lmo1421	CholTqMnF1	5′-CCA CTG ACA ACT GGA ACC ATT TAT A-3′
	CholTqMnR1	5′-GAA AGA GCG CAA TTT GTT GTA AAA-3′
bsh	HMMlmo2067-F	5′-GGC CTT AGT ATG GCA GGA CTC A-3′
	HMMlmo2067-R	5′-CAC ATT GTC CTT ACC TTC TGC AAA-3′
rpoB	RpoBTqMnF1	5′-TGT AAA ATA TGG ACG GCA TCG T-3′
	RpoBTqMnR1	5′-GCT GTT TGA ATC TCA ATT AAG TTT GG-3′

Table 3. Promoter sequences for σ^B-dependent genes *opuCA*, *lmo1421* and *bsh* in comparison with previously identified σ^B-dependent promoters from various organisms

Organism	Gene/operon	'−35_____−10'	Position*	Protein function
L. monocytogenes	*opuCA*	GTTTAA--N 14--GGGAAA	−58	Osmoprotection
L. monocytogenes	*lmo1421*	GGAATA--N 15--GGGTAA	−85	Osmoprotection (putative)
L. monocytogenes	*bsh*	GTTTTA--N 13--GGGTAC	−40	Conjugated BSH
L. monocytogenes	*sigB*	GTTTTA--N 14--GGGTAA	−37	Alternative sigma factor
L. monocytogenes	*prfA* (P2)	GTTACT--N 14--GGGTAT	−39	Transcriptional activator
B. subtilis	*sigB*	GTTTAA--N 14--GGGTAT	−41	Alternative sigma factor
S. aureus	*sigB*	GATTAG--N 14--GGGTAT	−42	Alternative sigma factor

*Position is the distance (in nt) between the start codon and the final base at −10.

to calculate activity units as described below. Fluorescence was measured in a Packard Fusion Instrument (Perkin Elmer) using an excitation filter of 360 nm and an emission filter of 460 nm. Fluorescence units were converted to picomoles of methylumbelliferone (MU^-) using a standard curve of known MU^- (Sigma) concentrations. GUS activities were expressed in activity units defined as picomoles of 4-MUG hydrolysed per millilitre of cells at $OD_{600}=1{\cdot}0$, per minute. For salt-stress experiments, percentage changes between GUS activities of the salt-exposed cells (0·5 M NaCl and 0·5 M KCl) and non-exposed cells (0·154 M NaCl) were calculated at each collection time point. The one-sample *t*-test was used to identify significant differences in activity units between samples exposed to the different test conditions at each time point. Normality of observations was satisfied using the Anderson–Darling test ($P<0{\cdot}05$). All statistical analyses were performed using MINITAB version 13 (Minitab).

RESULTS

σ^B-dependent expression of *opuCA*, *lmo1421* and *bsh*

Putative σ^B-dependent promoters upstream of *opuCA*, which encodes a component of an osmoprotectant ABC (ATP binding cassette) transporter (Fraser *et al.*, 2000), and upstream of *lmo1421*, which encodes a putative ABC transporter (Fraser *et al.*, 2003), had been identified previously by sequence analyses. Our group applied a Hidden Markov model to identify putative σ^B-dependent promoters (Kazmierczak *et al.*, 2003) in the published *L. monocytogenes* EGD genome (Glaser *et al.*, 2001). The results of the search confirmed the presence of predicted σ^B-dependent promoters upstream of *opuCA* and *lmo1421* and identified a predicted σ^B-dependent promoter upstream of *bsh*. RACE (Rapid Amplification of cDNA Ends) PCR analysis performed in our laboratory confirmed the existence and location of these σ^B-dependent promoters upstream of *opuCA*, *lmo1421* and *bsh* in *L. monocytogenes* 10403S (Kazmierczak *et al.*, 2003). All three promoters exhibit high similarity to previously identified σ^B-dependent promoters in *L. monocytogenes*, *B. subtilis* and *S. aureus* (Table 3). RT-PCR primers targeting *opuCA*, *lmo1421* and *bsh* were designed to allow semi-quantitative evaluation of mRNA expression levels for these three genes. In addition, RT-PCR primers were designed for amplification of *rpoB*, which does not exhibit an upstream, predicted σ^B-dependent promoter. RT-PCR on total RNA isolated from stationary-phase *L. monocytogenes* wild-type and Δ*sigB* cells clearly demonstrated that *opuCA*, *lmo1421* and *bsh* transcript levels are much higher in the *L. monocytogenes* wild-type strain (10403S) than in the Δ*sigB* strain (Fig. 1). RT-PCR amplification of *rpoB* showed visually indistinguishable *rpoB* transcript levels for both stationary-phase wild-type and Δ*sigB L. monocytogene*s cells.

The *opuCA–gus* fusion strains FSL S1-063 (wild-type) and FSL S1-059 (Δ*sigB*) were used to independently measure

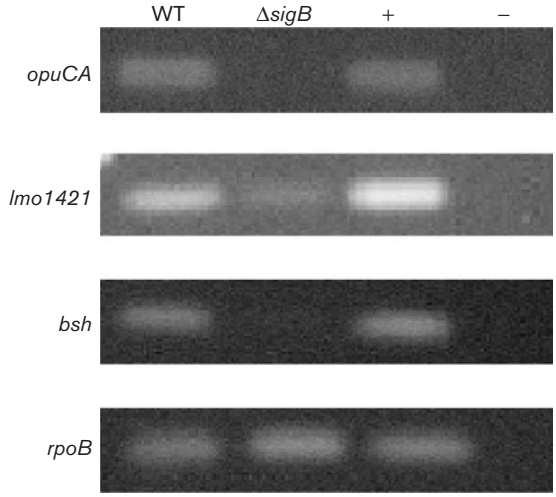

Fig. 1. Agarose gel electrophoresis of RT-PCR for *opuCA*, *lmo1421*, *bsh* and *rpoB* using 50 ng total RNA from stationary-phase ($OD_{600}=0{\cdot}8+1$ h) *L. monocytogenes* wild-type (WT) and Δ*sigB* strains. Also shown for each gene are PCR-positive (+) and -negative (−) control reactions using genomic DNA. Each image is one representative of at least two independent RT-PCR experiments performed. Gel electrophoresis of RT-negative reactions, in which Superscript II enzyme was omitted (data not shown), consistently demonstrated the absence of genomic DNA contamination.

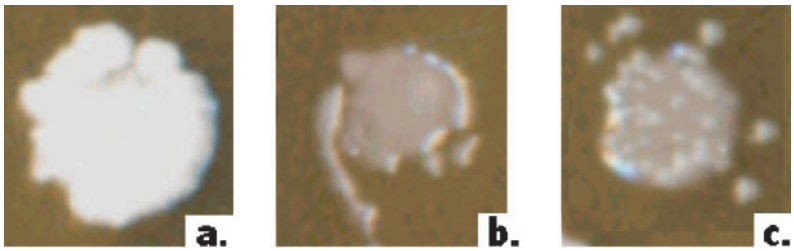

Fig. 2. Culture growth on MRS agar containing 0·5 % glycodeoxycholic acid for (a) *L. monocytogenes* wild-type strain 10403S, (b) *L. monocytogenes* Δ*sigB* strain FSL A1-254 and (c) *L. innocua* wild-type strain DD680 following incubation at 37 °C for 48 h. The heavy white precipitate of free bile acids in (a) demonstrates bile acid hydrolase activity in the wild-type strain. The absence of white precipitates in (b) and (c) indicates the absence of bile acid hydrolase activity in both the Δ*sigB* and the *L. innocua* strains.

expression of *opuCA* in the presence and absence of an intact *sigB*, respectively. At stationary phase, strain FSL S1-063 exhibited a GUS activity of 754 pmol MU^- ml^{-1} min^{-1} $(log\ c.f.u.)^{-1}$, while the *sigB* null mutant strain showed virtually no GUS activity [4·5 pmol MU^- ml^{-1} min^{-1} $(log\ c.f.u.)^{-1}$].

BSH assay

To confirm the role of σ^B in expression of *bsh*, which encodes a conjugated BSH (Dussurget *et al.*, 2002), *L. monocytogenes* wild-type 10403S, *L. innocua* DD608 (as a negative control) and the Δ*sigB* strain FSL A1-254 were spotted onto MRS medium agar containing 0·5 % glycodeoxycholic acid. A heavy white precipitate of free bile salt was observed only around the *L. monocytogenes* wild-type strain, but not around the Δ*sigB* or the *L. innocua* strains, indicating the presence of BSH activity only in the wild-type strain, but not in the others (Fig. 2).

Growth-phase-dependent expression of *opuCA*

The growth-phase dependence of *opuCA* expression, as determined by GUS reporter fusion assay, has been reported previously (Ferreira *et al.*, 2003). Briefly, *opuCA* expression increases in a growth-phase-dependent fashion as wild-type *L. monocytogenes* 10403S approaches stationary phase. *opuCA*-directed GUS activity is essentially abolished in a Δ*sigB* strain (Ferreira *et al.*, 2003). In this report, RT-PCR was used as an independent strategy for examining the role of σ^B in *L. monocytogenes opuCA* expression throughout growth and in stationary phase. *opuCA* transcript levels for *L. monocytogenes* 10403S increased in a growth-phase-dependent manner, as assessed by the increase in RT-PCR product band intensity when RNA was harvested from wild-type cells at OD_{600} values of 0·4, 0·8 and 0·8 + 1 h during growth in BHI (Fig. 3). No RT-PCR product was visible for the *opuCA* RT-PCR from RNA isolated from the *L. monocytogenes* Δ*sigB* strain grown to an OD_{600} value of 0·8 + 1 h, further confirming the σ^B-dependent expression of *opuCA*. The products from the *rpoB* RT-PCRs from *L. monocytogenes* wild-type and Δ*sigB* cells exhibited consistently strong bands under all conditions (Fig. 3). A semi-quantitative analysis, in which the band intensities for each *opuCA* RT-PCR were normalized to *rpoB* band intensities at corresponding collection time points, further confirmed the increase in relative *opuCA* expression throughout growth. Relative *opuCA* RT-PCR band intensities increased from 0·67 at $OD_{600} = 0·4$, to 0·83 at $OD_{600} = 0·8$, to 1·15 at $OD_{600} = 0·8 + 1$ h.

Salt-stress experiments

RT-PCR and *gus* reporter fusion strategies were also used to monitor induction of selected σ^B-dependent genes after exposure to salt-stress conditions. RT-PCR was used to monitor expression patterns of *opuCA*, *lmo1421* and *bsh* in the *L. monocytogenes* wild-type 10403S strain following exposure to salt stress (0·5 M KCl) as compared to a physiological salt concentration (0·121 M KCl) environment. Total RNA for RT-PCR analysis was obtained from exponential-phase bacterial cells exposed to either 0·5 or 0·121 M KCl for 5, 10 or 15 min. RT-PCR product band intensities for these genes were first normalized to RT-PCR band intensities for *rpoB*, which was used as a control gene. Normalized RT-PCR band intensities for the three σ^B-dependent target genes were then used to calculate relative

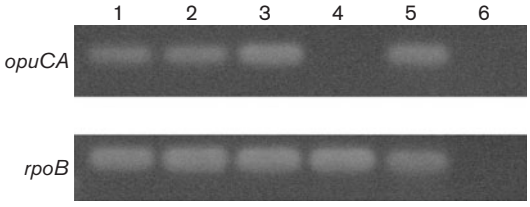

Fig. 3. Agarose gel electrophoresis of *opuCA* and *rpoB* RT-PCR products obtained from 50 ng RNA collected at three points during growth, using cells grown in BHI. Lanes: 1–3, RT-PCR products for *L. monocytogenes* wild-type strain using RNA collected at OD_{600} values of 0·4 (lane 1), 0·8 (lane 2) and 0·8 + 1 h (lane 3); 4, RT-PCR products for Δ*sigB* strain using RNA collected at $OD_{600} = 0·8 + 1$ h; 5, PCR-positive control performed on genomic DNA from the wild-type strain for *opuCA* and *rpoB*; 6, PCR-negative control, performed using water instead of genomic DNA. Control RT-PCRs without added reverse transcriptase were also performed for experiments represented by lanes 1–4 and showed no visible amplification products (not shown).

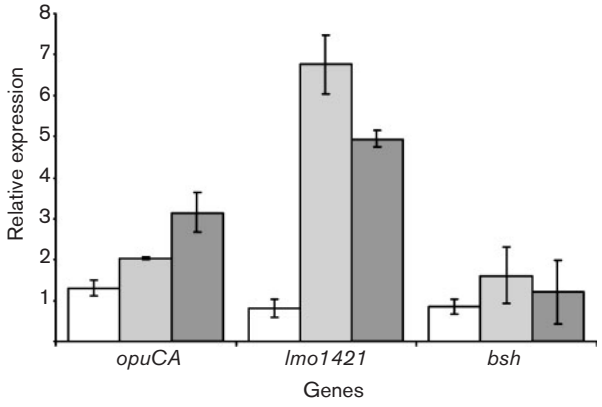

Fig. 4. Mean relative expression of *opuCA*, *lmo1421* and *bsh* in the *L. monocytogenes* wild-type strain exposed to 0·121 or 0·5 M KCl. Expression was determined by RT-PCR using RNA collected at 5 min (open bars), 10 min (light-grey bars) and 15 min (dark-grey bars) after exposure to 0·121 or 0·5 M KCl. Each RT-PCR used 50 ng total RNA. Products from *opuCA*, *lmo1421* and *bsh* expression were normalized to *rpoB*. Two independent RT-PCR experiments for *opuCA* and *lmo1421* and three independent RT-PCR experiments for *bsh* are shown. Bars indicate data range for *opuCA* and *lmo1421* and standard deviation for *bsh*. Relative expression equals induced expression divided by baseline expression.

induction coefficients, which represented the normalized RT-PCR band intensity for RNA from exposed cells (0·5 M KCl) relative to the normalized RT-PCR band intensity for the non-exposed cells (0·121 M KCl) collected at the same time point. Fig. 4 shows the results from two independent salt-stress experiments for the *opuCA* and *lmo1421* RT-PCRs and three independent salt-stress experiments for the *bsh* RT-PCRs. While these RT-PCR data show a consistent pattern of increased *opuCA* and *lmo1421* mRNA levels after exposure to 0·5 M KCl, we did not observe a reproducible increase in *bsh* mRNA levels following exposure to 0·5 M KCl.

The *opuCA–gus* reporter fusion strain FSL S1-063 was used to measure *opuCA* expression over time following exposure to salt stress (0·5 M NaCl, 0·5 M KCl). GUS activities were determined for bacterial cells collected after 15, 30, 60 and 120 min of exposure to salt stress (0·5 M NaCl, 0·5 M KCl) or to control conditions (0·154 M NaCl). Data from each of three independent experiments were used to calculate the relative GUS activities for exposed cells at each time point as compared to GUS activities for non-exposed cells. Fig. 5 shows the mean relative activities calculated from all three experiments. Significant differences ($P<0·05$) were observed following 15 and 30 min exposure to 0·5 M NaCl, when compared to non-induced cells (Fig. 5). Similarly, cells exposed to 0·5 M KCl for 15 min exhibited a significantly ($P<0·05$) higher GUS activity relative to non-exposed cells.

DISCUSSION

The alternative sigma factor σ^B plays an important role in the general stress response of the food-borne pathogen *L. monocytogenes* (Becker *et al.*, 2000; Wiedmann *et al.*, 1998). Although *sigB* null mutants have been characterized phenotypically (Becker *et al.*, 1998, 2000; Ferreira *et al.*, 2001; Nadon *et al.*, 2002; Wiedmann *et al.*, 1998), only three putative σ^B-dependent promoter regions (e.g. promoters for *rsbV*, *opuC* and *lmo1421*) had been described previously in *L. monocytogenes* (Becker *et al.*, 1998; Fraser *et al.*, 2003). While a putative σ^B-dependent promoter has been found upstream of *betL*, which encodes a sodium-dependent secondary betaine transporter, both reporter fusion and preliminary RT-PCR experiments have shown that *betL* expression is not σ^B-dependent (Fraser *et al.*, 2003). Stress-responsive induction has been demonstrated only for *rsbV* (Becker *et al.*, 1998) and upon entry into stationary phase for *opuCA* (Ferreira *et al.*, 2003). The objectives of this research

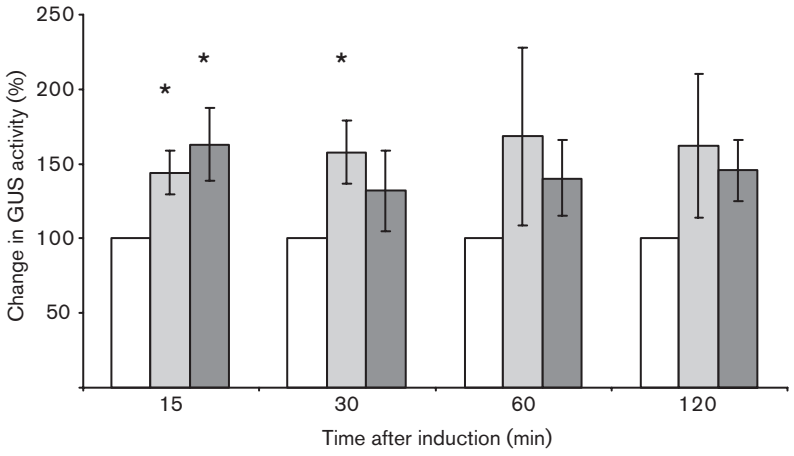

Fig. 5. Relative GUS activities for the *opuCA–gus L. monocytogenes* wild-type strain following exposure to 0·5 M NaCl or to 0·5 M KCl. The data shown represent the means of three independent experiments; bars indicate standard deviations. A one-sample *t*-test was used to identify significant differences in relative GUS activities between cells grown under control conditions (0·154 M NaCl) and cells exposed to salt-stress conditions at a given time point ($P<0·05$); GUS activity values significantly different from the control at a given time point are indicated by * in the figure. Normality of observations was satisfied using the Anderson–Darling test. Open bars, control; light-grey bars, 0·5 M NaCl; dark-grey bars, 0·5 M KCl.

were to confirm the σ^B-dependent expression of three putative σ^B-dependent genes (*opuCA*, *lmo1421* and *bsh*) using RT-PCR and reporter fusions and to monitor the induction and expression patterns of these genes under specific stress conditions, including entry into stationary phase and exposure to salt stress. We confirmed the σ^B dependence of these genes, each of which contributes to the *L. monocytogenes* general stress response, to virulence, or to both. Our data provide evidence for a broad role of σ^B in *L. monocytogenes* virulence and stress response and also provide new insight into expression profiles of selected members of the *L. monocytogenes* σ^B regulon.

Identification of σ^B-dependent genes and promoters

Semi-quantitative RT-PCR and *opuCA–gus* reporter fusions confirmed *opuCA*, *lmo1421* and *bsh* as members of the *L. monocytogenes* σ^B regulon. Previous investigations by Fraser and co-workers identified a putative σ^B-dependent promoter sequence upstream of the *opuC* operon (Fraser *et al.*, 2000) and *lmo1421* and provided initial evidence for σ^B-dependent transcription of these genes (Fraser *et al.*, 2003). Confirmation of *opuCA* and *lmo1421* as σ^B-dependent genes further illustrates the importance of σ^B in regulating transcription of osmotic stress genes in *L. monocytogenes*. In *L. monocytogenes*, OpuC is one of several compatible solute transporters (e.g. transporters encoded by *betL*, *gbu*) which confer increased osmotolerance to *L. monocytogenes* under osmotic stress environments (Fraser *et al.*, 2000; Ko & Smith, 1999; Sleator *et al.*, 1999). For example, under chill- and salt-stress conditions (0·5 M KCl), an *opuCB* mutant strain of *L. monocytogenes* 10403S exhibited a reduced ability to accumulate carnitine as compared to the wild-type strain (Angelidis *et al.*, 2002). *lmo1421* is predicted to encode the ATPase subunit of another ABC compatible solute (choline) transporter, based on sequence similarities to known transporter genes in *L. monocytogenes* and *B. subtilis* (Fraser & O'Byrne, 2002). Based on the high sequence homology with the *B. subtilis opuBA*, which encodes a choline transporter, other groups also have referred to *lmo1421* as *opuBA* (Sleator *et al.*, 2003; Wemekamp-Kamphuis *et al.*, 2002). OpuB has been demonstrated in *B. subtilis* to contribute to choline uptake (Kappes *et al.*, 1999) and thus is likely to contribute to osmotolerance in *L. monocytogenes*, although Sleator *et al.* (2003) proposed a possible role for OpuB in carnitine uptake in *L. monocytogenes* strain LO28. The demonstrated σ^B dependence of *opuCA* and *lmo1421* is consistent with the previous observation that a *L. monocytogenes sigB* null mutant shows a reduced ability to accumulate betaine and carnitine under osmotic- and cold-stress conditions (Becker *et al.*, 1998, 2000). Interestingly, *opuE*, which also encodes an osmoprotectant, is regulated by σ^B in *B. subtilis* (von Blohn *et al.*, 1997). In combination, these observations support a broad role for σ^B in regulation of osmolyte uptake systems in low-G + C-content Gram-positive bacteria.

In addition to *opuCA* and *lmo1421*, we also experimentally identified and confirmed *bsh* as a σ^B-dependent gene using a semi-quantitative RT-PCR approach. *bsh*, which encodes a conjugated BSH, was first identified in *L. monocytogenes* by Dussurget *et al.* (2002). Using the assay described by Dussurget *et al.* (2002), we demonstrated that only the *L. monocytogenes* wild-type strain hydrolysed bile salt, while no visually apparent bile salt hydrolysis was observed in the Δ*sigB* strain (Fig. 2). Thus, we phenotypically confirmed the importance of an intact *sigB* on expression of BSH activity in *L. monocytogenes*. Since a functional *bsh* was shown to be required for full virulence in guinea pig infections (Dussurget *et al.*, 2002), our results provide evidence for a role of σ^B in virulence gene expression and virulence in *L. monocytogenes*. The role of *bsh* as a virulence gene is further supported by the observation that its expression is regulated by PrfA, a general activator of virulence gene expression in *L. monocytogenes* (Dussurget *et al.*, 2002).

In combination with other studies, our results provide further evidence for a broad role of *L. monocytogenes* σ^B during host–pathogen interactions (Milohanic *et al.*, 2003). Specifically, previous work has shown that σ^B directly contributes to the regulation of *prfA* transcription (Nadon *et al.*, 2002), and that both *opuCA* and *bsh* contribute to intra-host survival by *L. monocytogenes* (Dussurget *et al.*, 2002; Sleator *et al.*, 2001; Wemekamp-Kamphuis *et al.*, 2002). An intact *opuC* was required for wild-type colonization of the mouse upper small intestine following peroral inoculation by *L. monocytogenes* LO28 (Sleator *et al.*, 2001; Wemekamp-Kamphuis *et al.*, 2002). Dussurget *et al.* (2002) described a reduction in the recovery of a *L. monocytogenes* EGD *bsh* null mutant as compared to its wild-type parent strain in guinea pig stools 48 h after intragastric inoculation (Dussurget *et al.*, 2002). While *bsh* appears to be specific to *L. monocytogenes* and is absent from the non-pathogenic *L. innocua* and *B. subtilis*, similarity searches for *opuCA* and *lmo1421* show that homologues for both these genes exist in both *B. subtilis* strain 168 and *L. innocua CLIP11262* (Glaser *et al.*, 2001). Thus, σ^B appears to be involved in the transcriptional regulation of both classical virulence genes (e.g. *bsh*, *prfA*) and of general stress-response genes, which are important for bacterial survival during host–pathogen interaction as well as for survival in non-host environments. During gastrointestinal passage, pathogens such as *L. monocytogenes* experience dramatic changes in environmental conditions, such as exposure to low pH in the stomach and to high osmolarity in the small intestine (Davenport, 1982). As σ^B fulfils functional roles in both virulence and general stress response and as the gastrointestinal environment encountered by *L. monocytogenes* during passage through a host imposes a set of physiological stresses on bacteria, we hypothesize that σ^B contributes to ensuring appropriate gene expression enabling bacterial survival under these conditions.

Growth-phase-dependent activation of σ^B

Through both a reporter fusion strategy (Ferreira *et al.*, 2003) and semi-quantitative RT-PCR, *opuCA* was demonstrated to be expressed in a growth-phase-dependent

manner. Maximal *opuCA*-directed GUS activity was observed at entry into the stationary phase of growth (Ferreira *et al.*, 2003), which generally corresponds with cellular exposure to nutrient-limiting and other stress conditions (Kolter *et al.*, 1993). Our results are consistent with those of Becker *et al.* (1998), who used primer extension analysis of the σ^B-dependent *rsbV* promoter to show induction of σ^B activity following entry into stationary phase. Studies in *B. subtilis* have also shown induction of σ^B activity upon entry into stationary phase (Boylan *et al.*, 1993; Varon *et al.*, 1996). Growth-phase-dependent expression of *opuCA* thus follows the typical σ^B-dependent expression profiles previously established for the σ^B-dependent *rsbV* in *L. monocytogenes* and for σ^B-dependent genes in other Gram-positive bacteria.

Induction of σ^B-dependent genes during salt stress

To further examine induction of σ^B-dependent genes under osmotic-stress conditions, we used *opuCA–gus* transcriptional gene fusions as well as semi-quantitative RT-PCR to monitor transcription of *opuCA*, *bsh* and *lmo1421*. Salt-stress conditions were selected for this study, as *L. monocytogenes* is likely to experience stress of this nature under food-processing conditions (e.g. in brines) and in food products associated with *L. monocytogenes* contamination (e.g. smoked fish and brined meat products) as well as during intra-host survival (Davenport, 1982). RT-PCR assays showed a rapid induction of *opuCA* and *lmo1421* under salt-stress conditions (i.e. within 5 min of salt-stress exposure). *opuCA* induction was also further confirmed by GUS reporter fusion assays. These data are consistent with preliminary primer extension experiments by Becker *et al.* (1998), who showed increased transcription of the σ^B-dependent *rsbV* promoter following salt stress in *L. monocytogenes*. Osmotic stress has also been shown to induce σ^B activity and transcription of the σ^B regulon in *B. subtilis* (Petersohn *et al.*, 2001). Interestingly, even though *bsh* clearly showed σ^B-dependent transcription, we were not able to observe consistent induction of *bsh* transcription following exposure of exponential-phase cells to salt-stress conditions. These results may indicate that *bsh* requires additional co-factors (other than the σ^B–RNA polymerase holoenzyme complex) for transcription induction, which would be consistent with the fact that BSH may only be required under specific environmental conditions (i.e. the presence of bile salts). The existence of σ^B-dependent genes that require additional σ^B-independent stress induction mechanisms has recently been demonstrated in *B. subtilis*. In fact, at least 24 of 125 genes in the *B. subtilis* regulon may require additional σ^B-independent stress-induction mechanisms (Petersohn *et al.*, 2001). Further studies on the regulation of *opuCA*, *lmo1421* and *bsh* will provide important insight into the complex mechanism(s) that likely govern expression of these stress-response genes and the *L. monocytogenes* σ^B regulon under different stress conditions, including those associated with intra-host environments.

Conclusions

L. monocytogenes has the unique ability to survive and grow under adverse environmental conditions including low pH, high osmolarity and low temperatures, and also to cause food-borne infections in mammalian hosts through survival and multiplication in the intracellular environment of host cells (Cole *et al.*, 1990; Farber & Peterkin, 1991; Lou & Yousef, 1999). Our data support an emerging model that proposes a link between environmental survival and virulence in the food-borne pathogen *L. monocytogenes*. We have identified and confirmed three σ^B-dependent genes, including two osmotolerance genes (*opuCA* and *lmo1421*) and a virulence gene (*bsh*). Together with the previous description of a σ^B-dependent promoter contributing to the transcription of *prfA* (Nadon *et al.*, 2002), which encodes a key positive regulator of virulence gene expression in *L. monocytogenes*, and the description of a set of PrfA-regulated genes preceded by putative σ^B-dependent promoters (Milohanic *et al.*, 2003), our data suggest that σ^B may broadly respond to host-associated bacterial stress conditions by directing appropriate gene expression patterns. Further use and refinement of the reporter fusion and RT-PCR tools for monitoring σ^B activity in *L. monocytogenes* described here will allow us to enhance our understanding of the contributions of σ^B to the pathogenesis of food-borne listeriosis.

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