

Development and optimization of an EGFP-based reporter for measuring the general stress response in *Listeria monocytogenes*

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A characteristic of the food-borne pathogen *Listeria monocytogenes* is its tolerance to the harsh conditions found both in minimally processed foods and the human gastrointestinal tract. This trait is partly under the control of the alternative sigma factor sigma B (σ^B). To study the mechanisms that trigger the activation of σ^B , and hence the development of stress tolerance, we have developed a fluorescent reporter fusion that allows the real-time activity of σ^B to be monitored. The reporter, designated $P_{lmo2230::egfp}$, fuses the strong σ^B -dependent promoter from the *lmo2230* gene (which encodes a putative arsenate reductase) to a gene encoding enhanced green fluorescence protein (EGFP). The reporter was integrated into the genomes of the wild-type strain *L. monocytogenes* EGD-e as well as two mutant derivatives lacking either *sigB* or *rsbV*. The resulting strains were used to study σ^B activation in response to growth phase and hyperosmotic stress. The wild-type was strongly fluorescent in stationary phase or in cultures with added NaCl and this fluorescence was abolished in both the *sigB* and *rsbV* backgrounds, consistent with the σ^B -dependency of the *lmo2230* promoter. During sudden osmotic upshock (addition of 0.5 M NaCl during growth) a real-time increase in fluorescence was observed microscopically, reaching maximal activation after 30 min. Flow cytometry was used to study the activation of σ^B at a population level by hyperosmotic stress during exponential growth. A strong and proportional increase in fluorescence was observed as the salt concentration increased from 0 to 0.9 M NaCl. Interestingly, there was considerable heterogeneity within the population and a significant proportion of cells failed to induce a high level of fluorescence, suggesting that σ^B activation occurs stochastically in response to hyperosmotic stress. Thus the $P_{lmo2230::egfp}$ is a powerful tool that will allow the stress response to be better studied in this important human pathogen.

Introduction

The gram positive bacterium *Listeria monocytogenes* is a facultative intracellular pathogen that is found widely in the environment (reviewed by Barbuddhe, 2009).¹ As a foodborne pathogen *L. monocytogenes* is able to survive and multiply in food during processing and/or storage. Key virulence factors are controlled by the positive regulatory factor A (PrfA) and also by the alternative RNA polymerase sigma factor SigB (σ^B).²⁻⁴ σ^B is involved in the initiation of transcription by recognizing σ^B promoter sequences and allowing the expression of a specific set of genes (σ^B regulon) when the appropriate conditions are encountered. A variety of stress and virulence-related phenotypes are associated with loss of the *sigB* gene, indicating that σ^B plays an important role both during infections and under stressful conditions (reviewed by O'Byrne, 2008).⁵ After the full genome sequence of *L. monocytogenes* became available⁶ a number of studies identified components of the σ^B regulon by looking at genes differentially

expressed in *L. monocytogenes* wild-type and corresponding *sigB* mutants using proteomic approaches^{7,8} or gene microarray technology.⁹⁻¹²

The mechanism responsible for sensing stress that leads to the activation of σ^B has not been fully characterized in *L. monocytogenes*. However, the signal transduction system controlling σ^B activity in *Bacillus subtilis*, has been well studied (reviewed by Hecker, 2007).¹³ A high level of conservation between the *sigB* operons in *B. subtilis* and *L. monocytogenes* suggests a shared mechanism of post-translational regulation of σ^B activity. In *B. subtilis* this mechanism involves partner switching of an anti sigma factor called RsbW between σ^B and anti-anti sigma factor called RsbV; when RsbW is sequestered by RsbV, σ^B is free to participate in transcription. In order to test this model and to develop an understanding of how σ^B is controlled in *L. monocytogenes*, molecular tools for measuring the activity status of σ^B in this pathogen are required. Therefore in the present study we aimed to develop an effective reporter for measuring σ^B activity

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in *L. monocytogenes*, which would then allow important questions to be addressed about the conditions that trigger the activation of σ^B and how this activity is modulated.

A number of transcriptional reporter systems based on bioluminescence and enzymatic assays have been used to study the regulation of transcription and the investigation of promoter activity in *L. monocytogenes*.¹⁴⁻¹⁶ However, the commonly used *lacZ*, *gus* or *lux* reporters are limited by the need for specific cofactors or exogenous substrates. In addition to these considerations we sought a system that could be detectable with multiple methods and not adversely affected by the measurement techniques or growth conditions. Fluorescent reporter systems containing green fluorescent protein (GFP), first described in *Aequorea*, jellyfish are extensively utilized in a wide range of other organisms including bacteria.¹⁷ As measurement of GFP activity in prokaryotes was reported to be affected reversibly by protein oxidation, the pH value of the medium and temperature,¹⁸ several chromophore variants of wild-type GFP (wtGFP) have been developed for use as a reporters under a wide range of conditions.¹⁹ For a reporter of σ^B activity the enhanced green fluorescent protein (EGFP) variant was selected, since it demonstrates 35-fold higher fluorescence and is more stable in terms of irreversible quenching and photo-bleaching, making the detection threshold of EGFP much lower than wtGFP.^{20,21} EGFP has a single, strong red shifted excitation peak at 488 nm which corresponds to the line of FITC optics and line of argon ion lasers used in many flow cytometers. In addition, elimination of the UV excitation enables utilization of EGFP in living cells under less harmful conditions due to lower energies involved in visualization. EGFP folding and chromophore formation is also faster²² and its structure was reported to be stable over osmolarity and temperature ranges.²³

One of the key factors in considering a successful reporter of σ^B activity in *L. monocytogenes* is the choice of an effective σ^B -dependent promoter. In the pioneering in vitro studies of sporulation in *B. subtilis* Haldenwang and Losick (1979)²⁴ used the *ctc* gene in their transcription assays, which led to the discovery of one of the first bacterial alternative sigma factor, σ^B . Since then *ctc* has become the first-choice reporter gene for σ^B activity studies in *B. subtilis*, where it has been used to investigate a whole range of different stress stimuli.²⁵⁻²⁷ The *ctc* gene, described as being positively regulated by σ^B in *L. monocytogenes*,¹¹ was also used in a transcriptomic approach to investigate blue and red light activation of σ^B in *L. monocytogenes*.²⁸ The study demonstrated that in a $\Delta sigB$ mutant there was still substantial *ctc* transcript levels, suggesting that in *L. monocytogenes* *ctc* transcription is not fully dependent on σ^B . The *sigB* gene itself is known to be autoregulated^{13,29} and has previously been used in *L. monocytogenes* as a reporter for the role of σ^B in biofilm formation.³⁰ However, the proposed regulation of transcription of eight-gene *sigB* operon based on *B. subtilis* and a transcriptomic study of *L. monocytogenes* suggest that *sigB* might also be transcribed in σ^B -independent manner from the σ^A promoter located upstream from *rsbR* and yielding the transcript for the whole operon including *rsbRSTU* followed by *rsbV*, *rsbW*, *sigB*

and *rsbX*²⁹ or within a 10 gene operon starting upstream from *lmo0887*.¹²

Our recently published work evaluated transcript levels of *sigB* and other σ^B -dependent genes as a reporter of σ^B activity.³¹ Out of four σ^B -dependent genes investigated (*sigB*, *opuCA*, *lmo2085*, *lmo2230*), expression of *lmo2230*, which encodes a putative arsenate reductase, was strongly σ^B -dependent and demonstrated the highest level of induction following osmotic stress. Selected studies on the σ^B regulon in various strains of *L. monocytogenes* over a range of different stress conditions^{9-11,32-35} have confirmed the high induction and strong σ^B -dependence of *lmo2230* expression. These studies also show that the σ^B -dependent activation of *lmo2230* transcription occurs in different strains and in response to different stresses. The σ^B promoter sequence of *lmo2230* was predicted with Hidden Markov model (HMM)-based searches and its transcriptional start site was confirmed by RACE-PCR.¹¹ *lmo2230* (designated as *arsC*) has been already successfully used among other σ^B -regulated genes as a reporter of σ^B activity after exposure to light.²⁸ However, this valuable and quantitative study used expensive and time consuming RNA extractions followed by RT-PCR assays. Here we describe the construction of a reporter fusion utilizing the promoter of *lmo2230* to monitor σ^B -dependent expression that will be quicker and more effective for testing over a wide range of conditions.

Materials and Methods

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *L. monocytogenes* strains were grown in Brain Heart Infusion (BHI) broth or agar (LabM) at 37°C unless otherwise stated. Cells were grown under continuous shaking from a starting OD₆₀₀ = 0.05 to OD₆₀₀ = 0.6 for exponential phase and up to ~16 h for stationary phase experiments. The volume of the medium occupied no more than 10% of the flask volume to ensure sufficient aeration. *Escherichia coli* strains used as intermediate vector hosts were grown in Luria Bertani (LB) broth or agar (LabM) at 30°C. Antibiotics were incorporated into the media as follows: Chloramphenicol (Cml) 10 $\mu\text{g ml}^{-1}$ for *L. monocytogenes*; Ampicillin (Amp) 100 $\mu\text{g/ml}$ for *E. coli*.³⁶

Construction of a *L. monocytogenes* EGD-e $\Delta rsbV$ mutant. *L. monocytogenes* EGD-e $\Delta rsbV$ mutant was constructed using the splicing by overlap extension (SOEing) PCR technique³⁷ followed by the construction of a shuttle vector containing the $\Delta rsbV$ deletion cassette. Primers A, B, C and D (COB385–388) were designed (based on EGD-e sequence published on <http://genolist.pasteur.fr/ListiList>) to amplify two fragments (AB and CD) of the gene to be deleted (Table 2). Primers A and D were designed to carry an *EcoRI* restriction endonuclease site at the 5' end of each primer. Primer C was designed to have its 5' region complementary to the 3' region of primer B. The intermediate vector pCR-XL-TOPO[®] (Invitrogen) was used to generate pEC04 carrying $\Delta rsbV$ deletion cassette prepared by SOEing. The $\Delta rsbV$ deletion cassette was subcloned into the pKSV7 shuttle vector yielding pEC06. The integration of pEC06 into

Table 1. Plasmids and strains used in this study

		Source or reference
PLASMIDS		
pKSV7 (pUC18 and pBD95 integrated shuttle vector for <i>E. coli</i> and <i>L. monocytogenes</i> carrying a temperature sensitive <i>oriC</i> from pE194)		36
pCR [®] -XL-TOPO [®]		Invitrogen [™]
pEC04 (pCR [®] -XL-TOPO [®] containing the Δ <i>rsbV</i> deletion cassette)		This study
pEC06 (pKSV7 containing Δ <i>rsbV</i> DNA deletion cassette)		This study
pBluescriptII-P _{<i>lmo2230::egfp</i>}		Eurofins MWG Operon
pKSV7-P _{<i>lmo2230::egfp</i>}		This study
STRAINS		
	Source or reference	Collection number
<i>E. coli</i> DH5 α /pKSV7	N. Freitag	COB082
One Shot [®] TOP10 Electrocomp [™] <i>E. coli</i>	Invitrogen [™]	COB267
<i>E. coli</i> TOP10/pBluescriptII-P _{<i>lmo2230::egfp</i>}	This study	COB515
<i>E. coli</i> TOP10/pKSV7-P _{<i>lmo2230::egfp</i>}	This study	COB516
<i>L. monocytogenes</i> EGD-e	K. Boor	COB261
<i>L. monocytogenes</i> EGD-e Δ <i>sigB</i>	K. Boor	COB262
<i>L. monocytogenes</i> EGD-e/pEC06	This study	COB392
<i>L. monocytogenes</i> EGD-e::pEC06	This study	COB393
<i>L. monocytogenes</i> EGD-e Δ <i>rsbV</i>	This study	COB411
<i>L. monocytogenes</i> EGD-e/pKSV7	This study	COB517
<i>L. monocytogenes</i> EGD-e/pKSV7-P _{<i>lmo2230::egfp</i>}	This study	COB476
<i>L. monocytogenes</i> EGD-e Δ <i>sigB</i> /pKSV7-P _{<i>lmo2230::egfp</i>}	This study	COB477
<i>L. monocytogenes</i> EGD-e Δ <i>rsbV</i> / pKSV7-P _{<i>lmo2230::egfp</i>}	This study	COB498
<i>L. monocytogenes</i> EGD-e::pKSV7-P _{<i>lmo2230::egfp</i>}	This study	COB491
<i>L. monocytogenes</i> EGD-e Δ <i>sigB</i> ::pKSV7-P _{<i>lmo2230::egfp</i>}	This study	COB495
<i>L. monocytogenes</i> EGD-e Δ <i>rsbV</i> ::pKSV7-P _{<i>lmo2230::egfp</i>}	This study	COB501

the *L. monocytogenes* chromosome was achieved by homologous recombination at 42°C (non permissive for pKSV7 replication) and finally the excision of the shuttle vector containing the wild type gene was obtained. Colonies were then screened for loss of the *rsbV* gene using PCR using primers COB470 and COB471 (Table 2) to confirm the elimination of *rsbV*. Primers COB330 and COB331 were used to verify that a fully intact neighbor *rsbW* gene existed in the Δ *rsbV* mutant and primers COB332 and COB333 provided similar finding for the *sigB* gene. Primers COB328 and COB329 were used to verify the *rsbV* deletion (312bp of 345bp).

Design and construction of P_{*lmo2230::egfp*} gene fusion strains. The reporter plasmid pKSV7-P_{*lmo2230::egfp*} (Fig. 1A) was designed to contain a fusion of the σ^B -dependent promoter region of *lmo2230* (located upstream of the start codon based on the full genome sequence for *L. monocytogenes* EGD-e) with a codon-optimised (with GENEius software) *egfp* (accession AY192024.1), the gene coding for enhanced green fluorescent protein (EGFP).²⁰ First, *egfp* was synthesized together with 443 bp sequence of the *lmo2230* promoter region upstream from its start codon¹¹ flanked by *Bam*HI and *Eco*RI restriction sites (together this gene fusion construct was designated P_{*lmo2230::egfp*}).

The gene fusion was synthesized by Eurofins MWG and inserted into pBluescriptII yielding pBluescriptII-P_{*lmo2230::egfp*}. The P_{*lmo2230::egfp*} fragment including the *Bam*HI and *Eco*RI restriction sites was amplified by PCR with Ultra High-Fidelity DNA Polymerase (Agilent Technologies) and M13 primers (Table 2) using pBluescriptII-P_{*lmo2230::egfp*} as a template. After digestion with *Bam*HI and *Eco*RI the P_{*lmo2230::egfp*} fragment was subcloned into pKSV7 using standard methodologies, generating plasmid pKSV7-P_{*lmo2230::egfp*}. This newly constructed plasmid was transformed into electrocompetent *L. monocytogenes* EGD-e wild type, Δ *sigB* and Δ *rsbV* derivative mutant strains as described earlier.³⁸ The transformants were selected on BHI agar plates containing 10 μ g ml⁻¹ of chloramphenicol, kept for 48 h at 30°C.

Chromosomal integration of the P_{*lmo2230::egfp*} reporter fusion. Homologous recombination was utilized to construct recombinant strains of *L. monocytogenes* EGD-e wild type, Δ *sigB* and Δ *rsbV* derivative mutants carrying single copies of the P_{*lmo2230::egfp*} reporter fusion integrated into the chromosome (Fig. 1B). The integration was achieved by growing the *L. monocytogenes* cells, electro-transformed with temperature sensitive pKSV7-P_{*lmo2230::egfp*} vector, on chloramphenicol plates, kept for 48 h at 42°C. At a non-permissive temperature the vector was

Table 2. Primers used in this study

Name	Sequence (5' to 3')	Anneal (°C)	Collection number
M13-F	CAGGAAACAGCTATGAC	50	COB162
M13-R	GTAAACGACGGCCAG	50	COB163
<i>rsbV</i> -CACC-F	CACCATGAATATTAGTATAGAAAATAAAAG	55	COB328
<i>rsbV</i> - <i>his₆</i> -R	TCAATGATGATGATGATGATGTGCATTGTCATTTCATTTTC	55	COB329
<i>rsbW</i> -CACC-F	CACCATGGCAACAATGCATGACAAAATTAC	55	COB330
<i>rsbW</i> - <i>His₆</i> -R	TCAGTGGTGGTGGATGATGATGGGTTGAGATACTTTTGGC	55	COB331
<i>sigB</i> -CACC-F	CACCATGCCAAAAGTATCTCAACCTG	55	COB332
<i>sigB</i> - <i>His₆</i> -R	TTAATGGTGTGGTGTGGTGTCTCCACTTCCTCATTCTG	55	COB333
<i>rsbV</i> SOE A	GAATTCGAAGGCTTACCACTGGG	58	COB385
<i>rsbV</i> SOE B	CATCACTTCACCCCATCTAATTTTT	58	COB386
<i>rsbV</i> SOE C	GGGGGTGAAGTGATGGTAGAGGGTAAATGAATGGC	58	COB387
<i>rsbV</i> SOE D	GAATTCAGGTTGAGATACTTTTGGC	58	COB388
<i>P_{BrsbV}</i> - <i>His</i> -F	CCGAATTCCTTTGAGTTGCATGATGACTTTACGC	60	COB470
<i>P_{BrsbV}</i> - <i>His</i> -R	GCGAATTCCTCAATGATGATGATGATGAGTGCATTGTTGCC	60	COB471
<i>lmo2229</i> -F	GCCTTGTCGCCATCTTTG	55	COB688
<i>egfp</i> -R	GGCCGTTTACATCTCCATC	55	COB689

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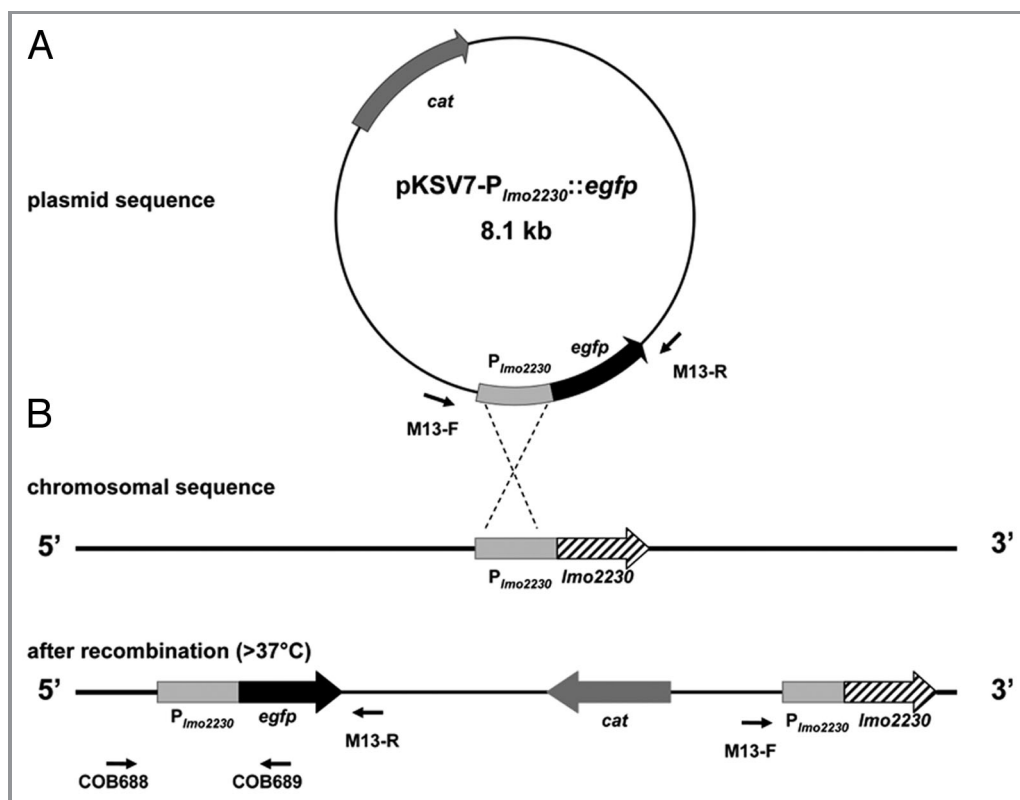


Figure 1. Design of the reporter plasmid pKSV7-*P_{lmo2230}::egfp* containing a fusion of the σ^B -dependent promoter region of *lmo2230* with *egfp* (A). Site-directed integration of the reporter fusion vector into the chromosome of *L. monocytogenes* EGD-e wild type and derivative integrants (B) occurring at homologous site of *lmo2230* promoter region (443 bp) after incubation at 42°C (non-permissive for autonomous plasmid maintenance in *L. monocytogenes*) in the presence of chloramphenicol selection.

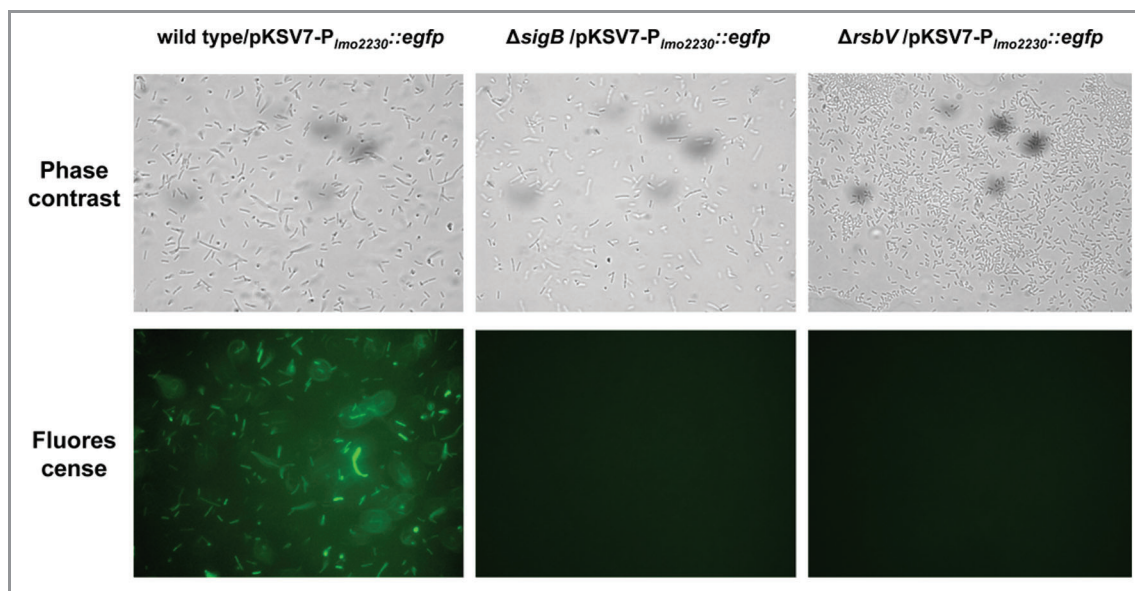


Figure 2. Fluorescence of *egfp*-containing cells of *L. monocytogenes* EGD-e wild type, $\Delta sigB$ and $\Delta rsbV$ mutants after transformation with pKSV7- $P_{Imo2230}$::*egfp*. Phase-contrast and fluorescence microscopy of corresponding fields were performed after 1 ml of stationary phase culture was centrifuged, resuspended in 50 μ l of sterile PBS and 5 μ l was smeared on the slides for visualization. Images are representative of a minimum of ten randomly selected fields captured for three biological replicates for each strain.

unable to replicate autonomously and integrated into the $P_{Imo2230}$ homologous region on the host chromosome. Fast growing colonies were sub-streaked five times onto fresh BHI agar with chloramphenicol, each time for an additional 24 h growth at 42°C. Integration was confirmed using PCR with COB688 and COB689 primers, which bind to defined regions in both the pKSV7- $P_{Imo2230}$::*egfp* plasmid and EGD-e genomic DNA (Table 2).

Microscopic quantification of EGFP fluorescence. To visualize fluorescence due to σ^B -promoter-driven EGFP expression, cells were grown in BHI or BHI supplemented with 0.5 M NaCl under continuous shaking to $OD_{600} = 0.6$. Then 1 ml of cells was immediately fixed in 1:1 volume of ice cold 1:1 (v/v) methanol/ethanol mixture for 10 min at -20°C maintaining fluorescence level similar to live cells when observed with microscopy. For quantitative fluorescence, 1 ml of fixed cells was diluted 1:10 by mixing with 9 ml of filter sterilised PBS and then cells were concentrated on 0.2 μ m pore, 10 mm diameter, black polycarbonate membrane filters (Millipore). For preliminary analyses, 1 ml of bacterial cells was centrifuged, resuspended in 50 μ l of sterile PBS and 5 μ l was smeared on the slides for visualization. Phase-contrast and fluorescence microscopy was performed using a Nikon Eclipse E600 microscope with a CCD camera attached for digital photography. Expression of EGFP was visualized at a fixed exposure time of 2 sec in a dark room using the B-2A filter covering GFP wavelengths together with 1/8 Neutral Density filter (ND8) for minimizing EGFP photobleaching. Relative fluorescence intensities and detectable cell counts were reported after automated image processing of multiple fields with ImageJ 1.44 software³⁹ with appropriate manipulations as described by others.^{40,41} For each experimental condition a minimum of three biological replicates were analyzed and ten randomly selected fields were captured.

Western blot analyses. Proteins were extracted using a sonication-based method³⁸ from 200 ml of stationary cultures grown for 16 h and the concentrations of protein extracts were determined by the RC DC Protein Assay Kit (BioRad). Extracts were normalized to 5 mg ml⁻¹ total protein concentrations and 10 μ l of these samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Western blot analyses were performed using chicken polyclonal anti-GFP antibody (Abcam) diluted 1:2,000 in 3% w/v skim milk and secondary antibody (HRP conjugated to goat anti-chicken IgY, Promega) diluted 1:20,000 in 3% w/v skim milk. Blots were viewed with a chemiluminescent substrate (SuperSignal[®] West Pico Chemiluminescent Kit, Pierce) captured in a dark room using a light sensitive film (Amersham Hyperfilm ECL, GE Healthcare).

Flow cytometry (FCM) analysis of the EGFP expressing cells. For FCM analysis of EGFP expression bacterial strains were grown from starting $OD_{600} = 0.05$ to $OD_{600} = 0.6$ in BHI or BHI supplemented with 0.3 M, 0.6 M, or 0.9 M NaCl. Cells were fixed in 1:1 volume of ice cold 1:1 (v/v) methanol/ethanol mixture for 10 min at -20°C. One ml of fixed cells was then harvested by centrifugation and resuspended in 0.5 ml of PBS. Quantification of single cell fluorescence was achieved by FCM analysis of 100 μ l of the suspension with a BD Accuri C6 flow cytometer (Accuri Cytometers, Inc.) using the following instrument settings: 488 nm blue laser excitation, FL1 533/30 nm (e.g., FITC/GFP) emission channel and fast 66 μ L/min flow rate from 96 round bottom plates (Sarstedt) and a minimum of 100,000 events for each sample recorded. The data collected were processed with BD CFlow[®] software to determine mean fluorescence values and plot side scatter and forward scatter values for two biological and three technical replicates.

Results

A $P_{lmo2230}::egfp$ reporter fusion generates strongly σ^B -dependent fluorescence. A fusion construct consisting of 443 bp of the *lmo2230* promoter region upstream from the *egfp* gene was synthesized and sub-cloned into pKSV7, generating a reporter plasmid designated pKSV7- $P_{lmo2230}::egfp$. This plasmid was then transformed into *L. monocytogenes* EGD-e (wild-type) as well as $\Delta sigB$ and $\Delta rsbV$ mutant derivatives of this strain. These transformants were investigated by fluorescent microscopy to determine whether EGFP was expressed in the three strains grown for 16 h in brain heart infusion (BHI) broth at 37°C. EGD-e wild-type cells harboring the pKSV7- $P_{lmo2230}::egfp$ plasmid were brightly fluorescent, although considerable heterogeneity in the level of EGFP fluorescence could be observed in the population (Fig. 2). Analysis of randomly selected microscopic fields with phase contrast and fluorescence microscopy suggests that some portion of wild-type cells transformed with pKSV7- $P_{lmo2230}::egfp$ were non-fluorescent or might express EGFP at a level not detectable with this method. In contrast to the wild-type, the $\Delta sigB$ mutant strain carrying pKSV7- $P_{lmo2230}::egfp$ showed no fluorescence, suggesting that σ^B is necessary for activation of *egfp* expression from the *lmo2230* promoter. Furthermore, the $\Delta rsbV$ strain harbouring this plasmid was also non-fluorescent, which is consistent with the proposed role of RsbV as an anti-anti sigma factor; in the absence of RsbV σ^B remains permanently sequestered by RsbW and is therefore unavailable to drive transcription from σ^B promoters.

To check if the lack of fluorescence of $\Delta sigB$, and $\Delta rsbV$ strains bearing the reporter plasmid was due to a detection limit of fluorescent microscopy we looked at *egfp* expression at the protein level. Western blotting using an anti-GFP antibody was performed on stationary cell extracts from wild-type, $\Delta sigB$, and $\Delta rsbV$ strains. EGFP was found to be expressed in wild-type only and neither full-length EGFP nor any indication of degraded protein bands were observed in the $\Delta sigB$ (Fig. 3A), and the $\Delta rsbV$ strains (Fig. 3B). These findings confirmed σ^B -dependent

expression of EGFP and also indicated an absence of RsbV-independent activation of σ^B under the conditions investigated.

Quantification of *lmo2230*-promoter-driven expression with fluorescent microscopy. In order to eliminate any artifacts that might be associated with a multi-copy plasmid-based reporter fusion (e.g., variable copy number or effects of antibiotics used for maintenance) the reporter fusion vector (Fig. 1A) was integrated into the genomes of *L. monocytogenes* EGD-e wild type, $\Delta sigB$ and $\Delta rsbV$ strains by homologous recombination (Fig. 1B). Microscopic comparison of wild type cells carrying either a chromosomal copy or a plasmid copy of the reporter fusion did not show a distinguishable difference in fluorescence (data not shown). As expected the $\Delta sigB$ and $\Delta rsbV$ integrants were not fluorescent. Integrant strains bearing single chromosomal copies of *egfp* could be grown without the need for chloramphenicol selection and consequently were more stable than plasmid bearing transformants, which required selection for plasmid maintenance.

It was possible to quantify the relative fluorescence levels in cultures growing under different experimental conditions using fluorescent microscopy and image analysis (Fig. 4). This method was based on detection of fluorescent cells using algorithms for automatic edge detection of bacteria, background subtraction and counting of the objects (Fig. 4B) and subsequently determining the mean fluorescent intensity of the objects detected (Fig. 4C). To evaluate this approach we examined the wild-type integrant strain in the presence of salt stress (0.5 M NaCl) and in stationary phase, conditions that σ^B is known to be activated by, and under which EGFP structure and its fluorescence are stable.²³ As expected EGFP expression was increased by salt in exponential phase and a higher fluorescence was observed in stationary phase when compared with the exponential phase of growth (Fig. 4). The salt induction in exponential phase could be visually observed in randomly selected microscopic fields (Fig. 4A; $n \geq 30$) and was further demonstrated by quantifying the number of fluorescent objects detected (Fig. 4B) and their relative fluorescence (Fig. 4C). However, the activation of σ^B by salt was not found to be statistically significant in stationary phase (Fig. 4C). Cells grown up to stationary phase of growth (-16 h) were more fluorescent than exponentially growing cells regardless of the salt addition. Thus the ability of the $P_{lmo2230}::egfp$ fusion to act as a reporter of σ^B activity is not adversely affected by genome integration.

Activation of σ^B is rapid after osmotic upshock. To determine whether our reporter system is able to detect quick changes of σ^B activity reported by earlier studies,^{31,32} the relative fluorescence was investigated after osmotic upshock. In contrast to steady-state growth experiments, cells were grown without NaCl supplementation up to $OD_{600} = 0.6$ then osmotically shocked by addition of solid NaCl (0.5 M) and samples were taken at suitable intervals from vigorously shaken cultures for microscopic observation (Fig. 5A) and fluorescence intensity quantification (Fig. 5B). A statistically significant increase ($p < 0.05$, Student's t-test) of relative fluorescence between osmotically shocked and untreated culture was observed from 10 min after the upshock (Fig. 5B). The reporter approach

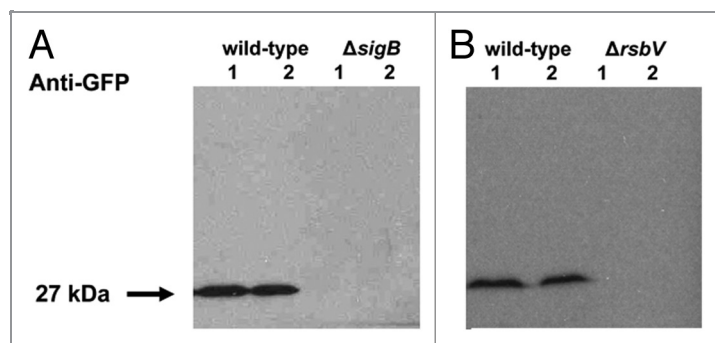


Figure 3. EGFP is expressed of *L. monocytogenes* EGD-e wild type but not in $\Delta sigB$ and $\Delta rsbV$ backgrounds. Western blot analyses were performed using protein extracts from stationary phase cultures (normalized to 5 mg ml⁻¹ total protein concentrations and 10 μ l of these samples separated by SDS-PAGE) with chicken polyclonal anti-GFP antibody (Abcam) HRP-conjugated goat anti-chicken antibody (Promega) and chemiluminescent detection.

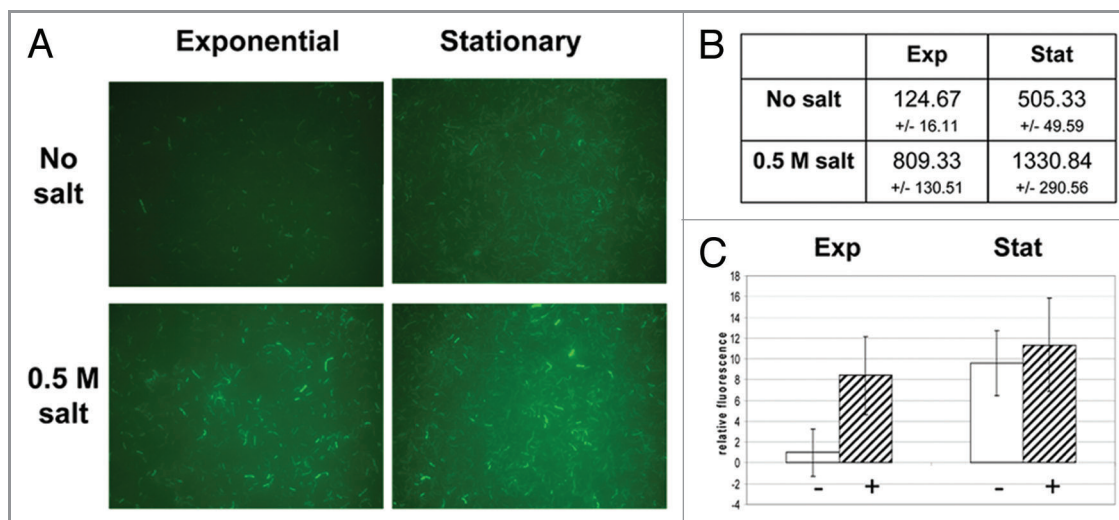


Figure 4. σ^B -dependent expression of EGFP is increased in *L. monocytogenes* EGD-e wild-type integrant in stationary phase and by osmotic stress. Cells of integrants bearing $P_{lmo2230}::egfp$ fusion were grown from starting $OD_{600} = 0.05$ up to either exponential ($OD_{600} = 0.6$) or stationary phase (16 h) in BHI or BHI supplemented with 0.5 M NaCl. A similar number of cells (equivalent to 1 ml of $OD_{600} = 0.6$ and diluted 1:2 by the fixing procedure) was diluted 1:10 in PBS and concentrated on 10 mm diameter, 0.2 μm pore polycarbonate membrane (Millipore) by filtration. For each experimental condition three biological replicates were analyzed and a minimum ten randomly selected fields of each membrane were captured with a CCD camera attached to Nikon Eclipse E600 with B-2A and ND8 filters used at a fixed exposure time of 2 sec with representative fields shown (A). Fluorescence levels of cells were quantified with automated image processing of microscopic fields by counting numbers of detectable particles (B) and their relative fluorescence intensities (C) with ImageJ 1.44 software with appropriate manipulations as described by others.^{39,40}

demonstrated an induction of σ^B activity with the highest variability up to 20 min after salt addition and then stable fluorescence subsequent to 30 min after treatment. Together these results show that a chromosomally integrated $P_{lmo2230}::egfp$ reporter fusion can reliably detect a rapid activation of σ^B following osmotic upshock.

Flow cytometry reveals heterogeneous activation of σ^B in the population. Digital image processing is cheap and straight forward but also time-consuming especially in terms of the investigation of multiple experimental conditions or for determining the proportion of the population that is fluorescent. For a more automated analysis of cells expressing EGFP flow cytometry (FCM) was utilized and evaluated in cultures with increasing concentrations of salt. FCM allows individual cells to be analyzed whereas other methods give average data for the whole population. When wild-type cells without a reporter fusion were analyzed by FCM the population was found to have a low level of intrinsic fluorescence (autofluorescence). This “background” fluorescence level was used to define the gate range used to detect EGFP expressing cells. When the wild-type $P_{lmo2230}::egfp$ fusion strain was analyzed, following aerobic growth to exponential phase ($OD_{600} = 0.6$) at 37°C, 27.8% of the particles detected had fluorescence levels that fell within the defined gate (Fig. 6). When a FCM analysis was performed on a culture of the $\Delta sigB$ mutant grown under the same conditions, no significant fluorescence was detected above the background level, indicating that σ^B was responsible for any EGFP expression detected in the wild type. When cultures were grown under conditions of hyperosmotic stress (NaCl concentrations of 0.3, 0.6 or 0.9 M) the wild-type reporter strain showed a clear increase in the

proportion of cells that were expressing EGFP, while no EGFP expression was detected in the $\Delta sigB$ mutant (Fig. 6). The proportion of cells expressing EGFP more than doubled as the NaCl concentration increased from 0 to 0.9 M, reaching 62.2% of the population at 0.9 M NaCl. The data presented are in line with our previous transcriptomic reports of σ^B activity being proportional to the extent of NaCl stress.³¹

The heterogeneity of fluorescence within the wild-type EGFP expressing population was significant; with ~38% of the population exposed to 0.9 M NaCl failing to induce fluorescence levels that fell within the predetermined gate (Fig. 6A). The gate was determined by defining the limit of autofluorescence of wild-type *L. monocytogenes*. However it was clear that there was an increase in the level of autofluorescence when cells were exposed to 0.9 M NaCl and that increase was σ^B -dependent (Fig. 6). This result indicates that there is some overlap between cellular autofluorescence and EGFP fluorescence and also shows that the range of σ^B activities within the population is considerable. The heterogeneity of σ^B activities within the population exposed to hyperosmotic stress raises new and interesting questions about how individual cells sense and respond to osmotic stress.

Discussion

In the present study, we developed a rapid EGFP-based reporter assay for measuring σ^B activity in *L. monocytogenes*. The system was carefully designed based on available literature to have the strong σ^B -dependent promoter of *lmo2230*, which ensured that cells were highly fluorescent following the activation of σ^B . The reporter approach showed increased σ^B activity in stationary

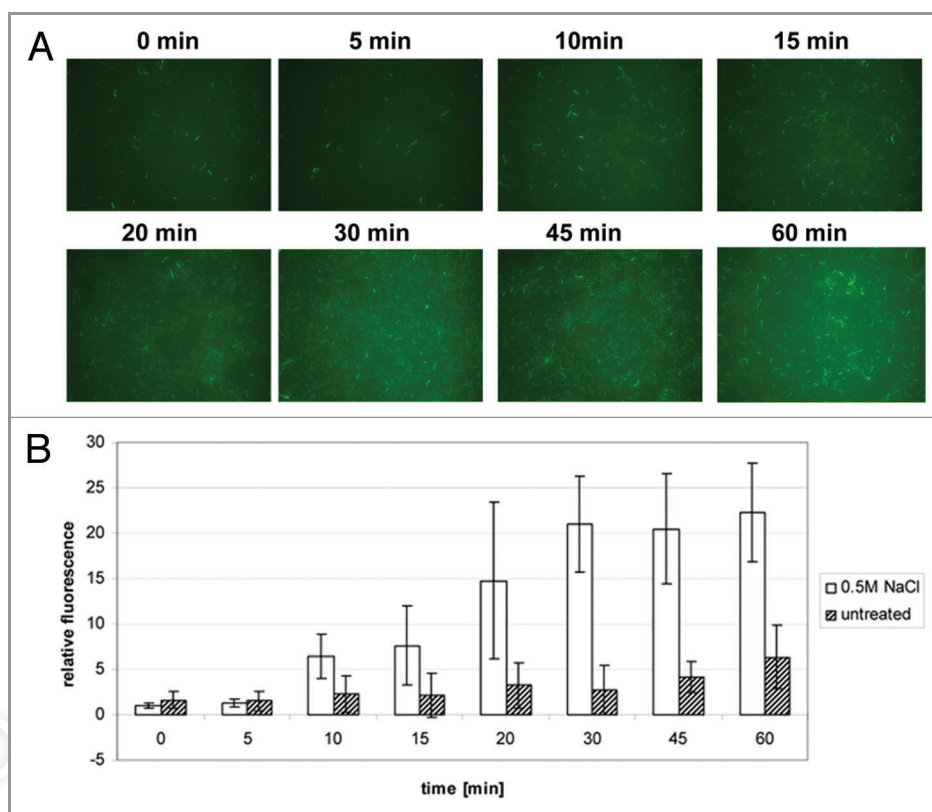


Figure 5. σ^B -dependent EGFP expression is induced after osmotic upshock in *L. monocytogenes* EGD-e wild-type $P_{lmo2230}::egfp$ integrant. Cells bearing $P_{lmo2230}::egfp$ fusion grown in BHI from $OD_{600} = 0.05$ up to $OD_{600} = 0.6$ when 0.5 M NaCl was added. Similar number of cells (equivalent to 1 ml of $OD_{600} = 0.6$ and diluted 1:2 by the fixing procedure) was taken at appropriate intervals from untreated and osmotically shocked cultures. Fixed cells were diluted 1:10 in PBS and concentrated on 10 mm diameter, 0.2 μm pore polycarbonate membrane (Millipore) by filtration. For each experimental condition three biological replicates were analyzed and a minimum of ten randomly selected fields were captured with a CCD camera attached to Nikon Eclipse E600 with B-2A and ND8 filters were used at a fixed exposure time of 2 s with representative fields shown (A). Fluorescence levels of cells were quantified with automated image processing of captured microscopic fields by counting relative fluorescence intensities (B) with ImageJ 1.44 software with appropriate manipulations as described by others.^{39,40}

phase and during hyperosmotic stress, which was applied either continuously or suddenly. Furthermore, an analysis by flow cytometry revealed that σ^B activation was heterogeneous in the population and proportional to the extent of stress. The σ^B -dependency of the reporter fusion was confirmed since strains lacking either the *sigB* or *rsbV* genes failed to induce EGFP expression under any condition tested. Together the data presented here demonstrate that the $P_{lmo2230}::egfp$ reporter is a useful tool that can be used to investigate the conditions and mechanisms that trigger the activation of σ^B in *L. monocytogenes*.

Expression of *lmo2230* was shown to be rapid and transient both after heat shock at 48°C, with a 10-fold induction observed after 3 min of heat stress³² and also 15 min after osmotic upshock when more than 160-fold higher levels of *lmo2230* in wild-type were reported comparing to unstressed cells at time zero.³¹ Taken together these findings give an opportunity for monitoring activation of σ^B in real time by following *lmo2230*-promoter-driven expression after a sudden change of environmental conditions. However, there are also some limitations in terms of reporting transient activation of σ^B with the EGFP-based reporter system caused by the high stability of the fluorescent protein under most

of the experimental conditions. The long half-life of EGFP (estimated at greater than 24 h)⁴² makes it impossible to observe a drop in σ^B activity after the removal of stress, while this decrease can be demonstrated by monitoring the transcript levels of *lmo2230* and other σ^B -regulated genes under similar conditions³¹ and after heat shock.³² Future versions of this reporter could include less stable GFP variants (with *ssrA* RNA tags recognized by housekeeping proteases)⁴² in order to overcome this limitation. When the time scale for σ^B activation was compared between the RNA based approach described earlier³¹ and the $P_{lmo2230}::egfp$ reporter (Fig. 5B), the maximal σ^B activation was evident approximately 15 min earlier when monitoring mRNA levels. It seems likely that this lag observed with microscopy is due to the time required for EGFP translation, protein folding and the chromophore maturation process.^{19,43} Overall, despite limitations related to the stability and maturation of EGFP, this reporter system should prove very useful for applications that require knowledge about the induction of σ^B activity in response to stress.

Another unique advantage of the $P_{lmo2230}::egfp$ reporter is that σ^B activation can be studied both at the single cell and the population levels, using microscopy and flow cytometry. Using

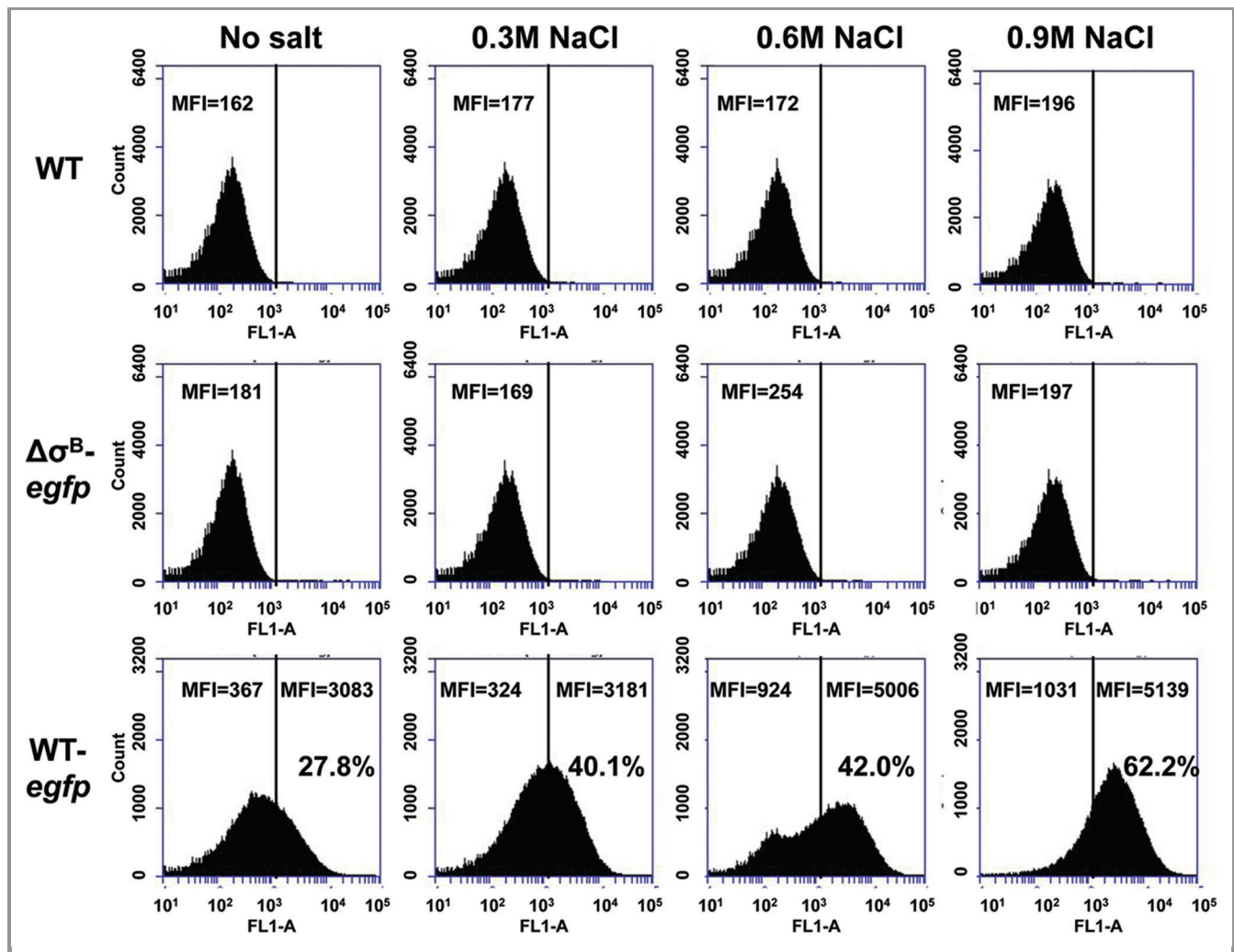


Figure 6. Heterogeneity of fluorescence within EGFP-expressing population and σ^B activation proportional to the extent of osmotic stress was revealed with FCM. Cells of parent wild-type strain (WT), wild-type- $P_{lmo2230}::egfp$ (WT- $egfp$) and $\Delta sigB$ - $P_{lmo2230}::egfp$ ($\Delta\sigma^B$ - $egfp$) derivative strains were grown from a starting $OD_{600} = 0.05$ up to $OD_{600} = 0.6$ in BHI (no salt) or BHI supplemented with 0.3 M, 0.6 M or 0.9 M NaCl. FCM was performed using BD Accuri C6 flow cytometer on fixed cells (concentration equivalent to 1 ml of $OD_{600} = 0.6$, fixed and resuspended in sterile PBS) for three technical replicates of two biological replicates for each strain and each experimental condition. Levels of intrinsic fluorescence (autofluorescence) were determined for wild-type cells without a reporter fusion (WT) and were used to define the gate range of EGFP expressing wild-type- $egfp$ strain population. Mean fluorescence intensities (MFI) for $egfp$ gate and particles located outside the gate (autofluorescence) were calculated by BD CFlow[®] software.

these approaches heterogeneity of the population and stochastic cell-to-cell variability of gene expression in *L. monocytogenes* can be studied in response to strictly defined stimuli. The flow cytometry data presented here indicate that cells from the same vigorously shaken liquid culture exhibit significant differences in fluorescence intensities (Fig. 6). This result indicates that σ^B activation does not occur uniformly in the population and further suggests that not all cells may perceive osmotic stress in the same way. Phenotypic variations in clonal populations have been investigated in other bacteria and partly explained by fluctuations in the amount of cellular components together with the noise and the asynchrony in gene expression.⁴⁴ It will be interesting in future studies to determine whether cell-cell differences in σ^B activation influences the survival of individual cells in foods or within the gastrointestinal tract.⁴⁵

In an $\Delta rsbV$ background no EGFP expression was detected from the $P_{lmo2230}::egfp$ reporter (Figs. 2 and 3B), consistent with the current model for σ^B activation, which proposes that RsbV acts as an anti-anti-sigma factor that facilitates σ^B activation when it interacts with the anti-sigma factor RsbW.¹³ Indeed there is genetic data from *L. monocytogenes* 10403S to support this model; *rsbV* and *sigB* mutants are found to have essentially the same phenotype, suggesting that RsbV is required for σ^B activation.⁴⁶ This highlights the potential use of the $P_{lmo2230}::egfp$ reporter in conducting studies on the regulation of σ^B . A key question that remains to be answered concerns the nature of the stress sensing mechanism. In *B. subtilis* a high molecular weight (~2MDa) sensory organelle called a stressosome is involved in sensing although the mechanism has not yet been elucidated. Stressosomes are also likely to exist in *L. monocytogenes*, since homologs

of all of the *B. subtilis* stressosome proteins are present in the genome, but their existence has not yet been established experimentally. By making targeted mutations in the predicted components of the stressosome it should be possible to use the $P_{lmo2230}::egfp$ reporter to determine which components are required for stress sensing and σ^B activation.

In summary, the $P_{lmo2230}::egfp$ fusion was developed and shown to be a reliable indicator of σ^B activity in *L. monocytogenes*, both during different phases of growth and under conditions of hyperosmotic stress. The tight σ^B -dependence of this promoter combined with the simplicity of detecting GFP fluorescence means that this reporter can be used for many applications in the study of the stress responses of this important food-borne pathogen.

References

- Barbuddhe SB, Chakraborty T. *Listeria* as an enteroinvasive gastrointestinal pathogen. *Curr Top Microbiol Immunol* 2009; 337:173-95; PMID:19812983; http://dx.doi.org/10.1007/978-3-642-01846-6_6
- Milohanic E, Glaser P, Coppée JY, Frangeul L, Vega Y, Vázquez-Boland JA, et al. Transcriptome analysis of *Listeria monocytogenes* identifies three groups of genes differently regulated by PrfA. *Mol Microbiol* 2003; 47:1613-25; PMID:12622816; <http://dx.doi.org/10.1046/j.1365-2958.2003.03413.x>
- Rauch M, Luo Q, Müller-Altröck S, Goebel W. SigB-dependent in vitro transcription of prfA and some newly identified genes of *Listeria monocytogenes* whose expression is affected by PrfA in vivo. *J Bacteriol* 2005; 187:800-4; PMID:15629954; <http://dx.doi.org/10.1128/JB.187.2.800-804.2005>
- Ollinger J, Wiedmann M, Boor KJ. SigmaB- and PrfA-dependent transcription of genes previously classified as putative constituents of the *Listeria monocytogenes* PrfA regulon. *Foodborne Pathog Dis* 2008; 5:281-93; PMID:18564909; <http://dx.doi.org/10.1089/fpd.2008.0079>
- O'Byrne CP, Karatzas KA. The role of sigma B (sigma B) in the stress adaptations of *Listeria monocytogenes*: overlaps between stress adaptation and virulence. *Adv Appl Microbiol* 2008; 65:115-40; PMID:19026864; [http://dx.doi.org/10.1016/S0065-2164\(08\)00605-9](http://dx.doi.org/10.1016/S0065-2164(08)00605-9)
- Glaser P, Frangeul L, Buchrieser C, Rusniok C, Amend A, Baquero F, et al. Comparative genomics of *Listeria* species. *Science* 2001; 294:849-52; PMID:11679669
- Wemekamp-Kamphuis HH, Wouters JA, de Leeuw PP, Hain T, Chakraborty T, Abec T. Identification of sigma factor sigma B-controlled genes and their impact on acid stress, high hydrostatic pressure, and freeze survival in *Listeria monocytogenes* EGD-e. *Appl Environ Microbiol* 2004; 70:3457-66; PMID:15184144; <http://dx.doi.org/10.1128/AEM.70.6.3457-3466.2004>
- Abram F, Su WL, Wiedmann M, Boor KJ, Coote P, Botting C, et al. Proteomic analyses of a *Listeria monocytogenes* mutant lacking sigmaB identify new components of the sigmaB regulon and highlight a role for sigmaB in the utilization of glycerol. *Appl Environ Microbiol* 2008; 74:594-604; PMID:18065622; <http://dx.doi.org/10.1128/AEM.01921-07>
- Raengpradub S, Wiedmann M, Boor KJ. Comparative analysis of the sigma B-dependent stress responses in *Listeria monocytogenes* and *Listeria innocua* strains exposed to selected stress conditions. *Appl Environ Microbiol* 2008; 74:158-71; PMID:18024685; <http://dx.doi.org/10.1128/AEM.00951-07>
- Hain T, Hossain H, Chatterjee SS, Machata S, Volk U, Wagner S, et al. Temporal transcriptomic analysis of the *Listeria monocytogenes* EGD-e sigmaB regulon. *BMC Microbiol* 2008; 8:20; PMID:18226246; <http://dx.doi.org/10.1186/1471-2180-8-20>
- Kazmierczak MJ, Mithoe SC, Boor KJ, Wiedmann M. *Listeria monocytogenes* sigma B regulates stress response and virulence functions. *J Bacteriol* 2003; 185:5722-34; PMID:13129943; <http://dx.doi.org/10.1128/JB.185.19.5722-5734.2003>
- Toledo-Arana A, Dussurget O, Nikitas G, Sesto N, Guet-Revillet H, Balestrino D, et al. The *Listeria* transcriptional landscape from saprophytism to virulence. *Nature* 2009; 459:950-6; PMID:19448609; <http://dx.doi.org/10.1038/nature08080>
- Hecker M, Pané-Farré J, Völker U. SigB-dependent general stress response in *Bacillus subtilis* and related gram-positive bacteria. *Annu Rev Microbiol* 2007; 61:215-36; PMID:18035607; <http://dx.doi.org/10.1146/annurev.micro.61.080706.093445>
- Shin JH, Brody MS, Price CW. Physical and antibiotic stresses require activation of the RsbU phosphatase to induce the general stress response in *Listeria monocytogenes*. *Microbiology* 2010; 156:2660-9; PMID:20558511; <http://dx.doi.org/10.1099/mic.0.041202-0>
- Riedel CU, Monk IR, Casey PG, Morrissey D, O'Sullivan GC, Tangney M, et al. Improved luciferase tagging system for *Listeria monocytogenes* allows real-time monitoring in vivo and in vitro. *Appl Environ Microbiol* 2007; 73:3091-4; PMID:17351089; <http://dx.doi.org/10.1128/AEM.02940-06>
- Ferreira A, Sue D, O'Byrne CP, Boor KJ. Role of *Listeria monocytogenes* sigma(B) in survival of lethal acidic conditions and in the acquired acid tolerance response. *Appl Environ Microbiol* 2003; 69:2692-8; PMID:12732538; <http://dx.doi.org/10.1128/AEM.69.5.2692-2698.2003>
- Southward CM, Surette MG. The dynamic microbe: green fluorescent protein brings bacteria to light. *Mol Microbiol* 2002; 45:1191-6; PMID:12207688; <http://dx.doi.org/10.1046/j.1365-2958.2002.03089.x>
- Hansen MC, Palmer RJ, Jr., Udsen C, White DC, Molin S. Assessment of GFP fluorescence in cells of *Streptococcus gordonii* under conditions of low pH and low oxygen concentration. *Microbiology* 2001; 147:1383-91; PMID:11320140
- Cubitt AB, Heim R, Adams SR, Boyd AE, Gross LA, Tsien RY. Understanding, improving and using green fluorescent proteins. *Trends Biochem Sci* 1995; 20:448-55; PMID:8578587; [http://dx.doi.org/10.1016/S0968-0004\(00\)89099-4](http://dx.doi.org/10.1016/S0968-0004(00)89099-4)
- Heim R, Cubitt AB, Tsien RY. Improved green fluorescence. *Nature* 1995; 373:663-4; PMID:7854443; <http://dx.doi.org/10.1038/373663b0>
- Patterson GH, Knobel SM, Sharif WD, Kain SR, Piston DW. Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. *Biophys J* 1997; 73:2782-90; PMID:9370472; [http://dx.doi.org/10.1016/S0006-3495\(97\)78307-3](http://dx.doi.org/10.1016/S0006-3495(97)78307-3)
- Iizuka R, Funatsu T, Uemura S. Real-time single-molecule observation of green fluorescent protein synthesis by immobilized ribosomes. *Methods Mol Biol* 2011; 778:215-28; PMID:21809209; http://dx.doi.org/10.1007/978-1-61779-261-8_14
- Gerena-López Y, Nolan J, Wang L, Gaigalas A, Schwartz A, Fernández-Repollet E. Quantification of EGFP expression on Molt-4 T cells using calibration standards. *Cytometry A* 2004; 60:21-8; PMID:15229854; <http://dx.doi.org/10.1002/cyto.a.20019>
- Haldenwang WG, Losick R. A modified RNA polymerase transcribes a cloned gene under sporulation control in *Bacillus subtilis*. *Nature* 1979; 282:256-60; PMID:116131; <http://dx.doi.org/10.1038/282256a0>
- Benson AK, Haldenwang WG. Regulation of sigma B levels and activity in *Bacillus subtilis*. *J Bacteriol* 1993; 175:2347-56; PMID:8468294
- Brigulla M, Hoffmann T, Krisp A, Völker A, Bremer E, Völker U. Chill induction of the SigB-dependent general stress response in *Bacillus subtilis* and its contribution to low-temperature adaptation. *J Bacteriol* 2003; 185:4305-14; PMID:12867438; <http://dx.doi.org/10.1128/JB.185.15.4305-4314.2003>
- Prágai Z, Harwood CR. Regulatory interactions between the Pho and sigma(B)-dependent general stress regulons of *Bacillus subtilis*. *Microbiology* 2002; 148:1593-602; PMID:11988534
- Ondrusch N, Kreft J. Blue and red light modulates SigB-dependent gene transcription, swimming motility and invasiveness in *Listeria monocytogenes*. *PLoS One* 2011; 6:e16151; PMID:21264304; <http://dx.doi.org/10.1371/journal.pone.0016151>
- Becker LA, Cetin MS, Hutkins RW, Benson AK. Identification of the gene encoding the alternative sigma factor sigmaB from *Listeria monocytogenes* and its role in osmotolerance. *J Bacteriol* 1998; 180:4547-54; PMID:9721294
- van der Veen S, Abec T. Importance of SigB for *Listeria monocytogenes* static and continuous-flow biofilm formation and disinfectant resistance. *Appl Environ Microbiol* 2010; 76:7854-60; PMID:20889779; <http://dx.doi.org/10.1128/AEM.01519-10>

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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31. Utratna M, Shaw I, Starr E, O'Byrne CP. Rapid, transient, and proportional activation of σ^B in response to osmotic stress in *Listeria monocytogenes*. *Appl Environ Microbiol* 2011; 77:7841-5; PMID:21890665; <http://dx.doi.org/10.1128/AEM.05732-11>
32. van der Veen S, Hain T, Wouters JA, Hossain H, de Vos WM, Abec T, et al. The heat-shock response of *Listeria monocytogenes* comprises genes involved in heat shock, cell division, cell wall synthesis, and the SOS response. *Microbiology* 2007; 153:3593-607; PMID:17906156; <http://dx.doi.org/10.1099/mic.0.2007/006361-0>
33. Chan YC, Boor KJ, Wiedmann M. SigmaB-dependent and sigmaB-independent mechanisms contribute to transcription of *Listeria monocytogenes* cold stress genes during cold shock and cold growth. *Appl Environ Microbiol* 2007; 73:6019-29; PMID:17675428; <http://dx.doi.org/10.1128/AEM.00714-07>
34. Chaturongakul S, Boor KJ. SigmaB activation under environmental and energy stress conditions in *Listeria monocytogenes*. *Appl Environ Microbiol* 2006; 72:5197-203; PMID:16885265; <http://dx.doi.org/10.1128/AEM.03058-05>
35. Oliver HF, Orsi RH, Ponnala L, Keich U, Wang W, Sun Q, et al. Deep RNA sequencing of *L. monocytogenes* reveals overlapping and extensive stationary phase and sigma B-dependent transcriptomes, including multiple highly transcribed noncoding RNAs. *BMC Genomics* 2009; 10:641; PMID:20042087; <http://dx.doi.org/10.1186/1471-2164-10-641>
36. Smith K, Youngman P. Use of a new integrational vector to investigate compartment-specific expression of the *Bacillus subtilis* spoIIM gene. *Biochimie* 1992; 74:705-11; PMID:1391050; [http://dx.doi.org/10.1016/0300-9084\(92\)90143-3](http://dx.doi.org/10.1016/0300-9084(92)90143-3)
37. Horton RM, Cai ZL, Ho SN, Pease LR. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. *Biotechniques* 1990; 8:528-35; PMID:2357375
38. Abram F, Starr E, Karatzas KA, Matlawska-Wasowska K, Boyd A, Wiedmann M, et al. Identification of components of the sigma B regulon in *Listeria monocytogenes* that contribute to acid and salt tolerance. *Appl Environ Microbiol* 2008; 74:6848-58; PMID:18806006; <http://dx.doi.org/10.1128/AEM.00442-08>
39. Collins TJ. ImageJ for microscopy. *Biotechniques* 2007; 43(Suppl):25-30; PMID:17936939; <http://dx.doi.org/10.2144/000112517>
40. Hamilton N. Quantification and its applications in fluorescent microscopy imaging. *Traffic* 2009; 10:951-61; PMID:19500318; <http://dx.doi.org/10.1111/j.1600-0854.2009.00938.x>
41. Selinummi J, Seppälä J, Yli-Harja O, Puhakka JA. Software for quantification of labeled bacteria from digital microscope images by automated image analysis. *Biotechniques* 2005; 39:859-63; PMID:16382904; <http://dx.doi.org/10.2144/000112018>
42. Andersen JB, Sternberg C, Poulsen LK, Bjorn SP, Givskov M, Molin S. New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Appl Environ Microbiol* 1998; 64:2240-6; PMID:9603842
43. Sniegowski JA, Lappe JW, Patel HN, Huffman HA, Wachter RM. Base catalysis of chromophore formation in Arg96 and Glu222 variants of green fluorescent protein. *J Biol Chem* 2005; 280:26248-55; PMID:15888441; <http://dx.doi.org/10.1074/jbc.M412327200>
44. Elowitz MB, Levine AJ, Siggia ED, Swain PS. Stochastic gene expression in a single cell. *Science* 2002; 297:1183-6; PMID:12183631; <http://dx.doi.org/10.1126/science.1070919>
45. Booth IR. Stress and the single cell: intrapopulation diversity is a mechanism to ensure survival upon exposure to stress. *Int J Food Microbiol* 2002; 78:19-30; PMID:12222634; [http://dx.doi.org/10.1016/S0168-1605\(02\)00239-8](http://dx.doi.org/10.1016/S0168-1605(02)00239-8)
46. Chaturongakul S, Boor KJ. RsbT and RsbV contribute to sigmaB-dependent survival under environmental, energy, and intracellular stress conditions in *Listeria monocytogenes*. *Appl Environ Microbiol* 2004; 70:5349-56; PMID:15345420; <http://dx.doi.org/10.1128/AEM.70.9.5349-5356.2004>

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