<table>
<thead>
<tr>
<th><strong>Title</strong></th>
<th>The development of reporters to measure the general stress response in Listeria monocytogenes: population and single cell studies on the activation of SigmaB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author(s)</strong></td>
<td>Utratna, Marta</td>
</tr>
<tr>
<td><strong>Publication Date</strong></td>
<td>2012-09-28</td>
</tr>
<tr>
<td><strong>Item record</strong></td>
<td><a href="http://hdl.handle.net/10379/3395">http://hdl.handle.net/10379/3395</a></td>
</tr>
</tbody>
</table>
The development of reporters to measure the general stress response in *Listeria monocytogenes*: population and single cell studies on the activation of $\sigma^B$

Submitted by:

Marta Utratna

Thesis submitted for a PhD Degree by Research at the National University of Ireland, Galway

Research was conducted in: Department of Microbiology
School of Natural Sciences,
College of Science,
National University of Ireland, Galway

Submission Date: September 2012
Supervisor of Research: Dr. Conor P. O’Byrne
I dedicate this thesis to my beloved husband, Krzysztof, for his constant support throughout everything, and his unconditional love.

I love you dearly.
Acknowledgements

This thesis would not have been possible without the guidance and the help of several people. I’d like to thank those individuals who, in one way or another, shaped my PhD experience and helped to make me the person I am today.

First and foremost, I’d like to express my utmost gratitude to my PhD supervisor, Dr. Conor O’Byrne, for providing me with this wonderful opportunity, and for his assistance in the completion of this study. Your mentoring was a perfect balance between demanding supervision, independence and freedom within my research. Thank you for your day-to-day feedback and discussions throughout the course of my project, during the writing for publications and the thesis itself. To work with you over the last four years has been a great pleasure.

I would also like to thank all the former and present team members of the COB lab, including Nina, Emily, Florence, Eoin, James, Sinéad, Andreas, Conor, Moira, Beth, Christy, Yinka and Kerrie, for creating a pleasant environment in the lab, for all the help and recommendations received during my research and presentations, and for your proofreading, as well as the celebration of little moments of glory. Your support, especially in my 3rd year, provided me with real strength. It is very much appreciated, and will never be forgotten.

To all the members of the Microbiology Department of NUIG – the staff, technicians, postgrads and students I worked with during practicals and projects – thank you for everything.

My deepest gratitude to our collaborators from REMEDI and NCBES, with special thanks to members of the Glycoscience Group and my 203 office colleagues, for their support at the writing stage.

To my Polish friends whom I met in Ireland (too many to be listed individually, but you know who you are), thank you so much for always being there to listen, for all science-related and random chats, our weekend trips, nights out and house parties. I already miss our morning coffees in Moffetts. I’m sure our paths will cross again!

I would also like to extend my deepest gratitude to my family: my parents Barbara and Stanislaw; in-laws Aniela and Tadeusz; my brothers Maciek and Michal; siblings-in-law Jarek, Piotrek, Ania, Pawel, Magda and Mateusz; their partners and children; my aunts, uncles and cousins; and my closest friends from home. Thank you all for believing in me, for great weddings and other meetings during summer holidays and Christmas breaks! Dziękuję Wam za wsparcie z daleka!

Finally, I would like to thank my other half, Krzysztof, who supported me at each step of the journey, for moving to Galway and for all wonderful years we have shared together.
TABLE OF CONTENTS

ABSTRACT ................................................................................................................ 5
PUBLICATIONS IN THIS THESIS: ................................................................. 6
LIST OF FIGURES ............................................................................................. 7
LIST OF TABLES ................................................................................................. 9
LIST OF APPENDICES ................................................................................... 10
LIST OF ABBREVIATIONS ............................................................................... 12

1. CHAPTER 1 – INTRODUCTION ..................................................................... 14
  1.1. LISTERIA MONOCYTOGENES – A SAPROPHYTIC ORGANISM AND OPPORTUNISTIC PATHOGEN ............................................................... 15
      1.1.1. Taxonomy of L. monocytogenes ........................................................... 15
      1.1.2. Incidence of L. monocytogenes in nature ...................................... 17
  1.2. L. MONOCYTOGENES WITHIN THE HOST ...................................... 19
      1.2.1. Disease manifestations ............................................................... 19
      1.2.2. The uptake of L. monocytogenes into eukaryotic cells ............ 21
      1.2.3. Intracellular life cycle ................................................................. 22
  1.3. REGULATORS OF GENE EXPRESSION IN L. MONOCYTOGENES ... 25
      1.3.1. Positive regulatory factor A (PrfA) .............................................. 26
          1.3.1.1. General characteristics and the role of PrfA ......................... 26
          1.3.1.2. Regulation of PrfA ............................................................... 27
              1.3.1.2.1. Transcriptional regulation of prfA expression .............. 27
              1.3.1.2.2. Translational regulation of prfA expression ............... 28
              1.3.1.2.3. Post-translational regulation of PrfA. ......................... 29
      1.3.2. Sigma factors of RNA Polymerase in L. monocytogenes .......... 30
      1.3.3. Other transcriptional regulators ................................................... 32
      1.3.4. Regulatory RNAs ....................................................................... 33
      1.3.5. Regulatory networks ................................................................... 35
  1.4. ALTERNATIVE SIGMA FACTOR SIGMA B IN B. SUBTILIS .......... 38
      1.4.1. Discovery and regulation of σ^B in B. subtilis ......................... 38
      1.4.2. Tracking σ^B activity in B. subtilis ............................................. 41
          1.4.2.1. Identification of genes reporting σ^B activity in B. subtilis ...... 41
          1.4.2.2. Modulation of σ^B activity in B. subtilis in response to stress .. 44
  1.5. SIGMA FACTOR σ^B IN L. MONOCYTOGENES ............................... 48
      1.5.1. The role of σ^B in stress response in L. monocytogenes ............ 48
          1.5.1.1. Osmotic stress response, and the role of σ^B ...................... 49
          1.5.1.2. The role of σ^B in response to other stresses .................... 52
          1.5.1.3. The role of σ^B in virulence ............................................... 56
          1.5.1.4. The role of σ^B in metabolism .......................................... 57
      1.5.2. The present model of regulation of σ^B in L. monocytogenes ...... 58
          1.5.2.1. Transcriptional regulation of sigB expression ................... 58
          1.5.2.2. Post-translational regulation of σ^B activity ....................... 60
  1.6. PROJECT AIMS ....................................................................................... 63

2. CHAPTER 2 ..................................................................................................... 65
Rapid, transient and proportional activation of σ^B in response to osmotic stress in Listeria monocytogenes ................................................................. 65
  2.1. ABSTRACT .............................................................................................. 66
  2.2. Note aricle ............................................................................................... 67
3.CHAPTER 3 .......................................................................................................... 82
Development and optimization of an EGFP-based reporter for measuring the
general stress response in Listeria monocytogenes ............................................. 82
3.1. ABSTRACT .................................................................................................... 83
3.2. INTRODUCTION .......................................................................................... 84
3.3. MATERIALS AND METHODS .................................................................... 87
  3.3.1. Bacterial strains, plasmids and growth conditions .................................... 87
  3.3.2. Construction of a L. monocytogenes EGD-e ΔrsbV mutant ....................... 87
  3.3.3. Design and construction of P_{lmo2230::egfp} gene fusion strains .......... 89
  3.3.4. Chromosomal integration of the P_{lmo2230::egfp} reporter fusion .......... 90
  3.3.5. Microscopic quantification of EGFP fluorescence ............................... 91
  3.3.6. Western blot analyses ........................................................................... 91
  3.3.7. Flow cytometry (FCM) analysis of the EGFP expressing cells .......... 92
3.4. RESULTS ....................................................................................................... 92
  3.4.1. A P_{lmo2230::egfp} reporter fusion generates strongly σ^B-dependent
        fluorescence ....................................................................................................... 92
  3.4.2. Quantification of lmo2230-promoter-driven expression with fluorescent
        microscopy .......................................................................................................... 95
  3.4.3. Activation of σ^B is rapid after osmotic upshock .................................. 96
  3.4.4. Flow cytometry reveals heterogeneous activation of σ^B in the population 98
3.5. DISCUSSION ............................................................................................... 100
3.6. ACKNOWLEDGEMENTS .......................................................................... 103

4.CHAPTER 4 ........................................................................................................ 106
Effects of growth phase and temperature on σ^B activity within a Listeria
monocytogenes population: Evidence for RsbV-independent activation of σ^B at
refrigeration temperatures .................................................................................... 106
4.1. ABSTRACT .................................................................................................. 107
4.2. INTRODUCTION ......................................................................................... 108
4.3. MATERIALS AND METHODS .................................................................. 110
  4.3.1. Growth conditions ................................................................................ 110
  4.3.2. Protein extraction ............................................................................... 112
  4.3.3. Polyclonal antisera generation ............................................................. 112
  4.3.4. Western blotting .................................................................................. 113
  4.3.5. Flow cytometry .................................................................................... 114
  4.3.6. Visualization of the fluorescence with microscopy ......................... 115
4.4. RESULTS ..................................................................................................... 115
  4.4.1. σ^B activity is not correlated with cellular levels of σ^B protein .......... 115
  4.4.2. σ^B is activated during exponential growth at both 37°C and 4°C ...... 117
  4.4.3. An alternative route of σ^B activation exists at 4°C in the absence of RsbV 120
4.5 DISCUSSION ............................................................................................... 123
  4.5.1. σ^B activity increases during exponential phase .............................. 123
  4.5.2. σ^B activity is not induced by low temperature in L. monocytogenes ..... 123
  4.5.3. RsbV-independent σ^B activation ....................................................... 124
4.6. Acknowledgements ..................................................................................... 125
5. CHAPTER 5 - SUMMARY DISCUSSION ...................................................... 128
5.1 Introduction .............................................................................................. 129
5.2. Evaluation of different methodological approaches for measuring $\sigma^B$ activity in L. monocytogenes .................................................................................. 129
5.3 Regulation of lmo2230 and its putative role in L. monocytogenes .......... 132
5.4 The role of stochasticity in the heterogeneous activation of $\sigma^B$ .......... 135
5.5. Stress sensing and signal propagation in the $\sigma^B$ regulatory pathway .... 138
5.6. Summary and future directions ............................................................... 141

6. CHAPTER 6 – REFERENCES ........................................................................ 143

7. CHAPTER 7 - APPENDICES .................................................................... 166
Appendix 1 ................................................................................................... 167
Appendix 2 ................................................................................................... 170
Appendix 3 ................................................................................................... 172
Appendix 4 ................................................................................................... 173
Appendix 5 ................................................................................................... 175
Appendix 6 ................................................................................................... 177
Appendix 7 ................................................................................................... 180
Appendix 8 ................................................................................................... 182
Appendix 9 ................................................................................................... 183
Appendix 10 .................................................................................................. 186
Appendix 11 .................................................................................................. 188
Appendix 12 .................................................................................................. 192
Appendix 13 .................................................................................................. 195
Appendix 14 .................................................................................................. 197
Appendix 15 .................................................................................................. 199
Appendix 16 .................................................................................................. 203
Appendix 17 .................................................................................................. 206
Appendix 18 .................................................................................................. 211
Appendix 19 .................................................................................................. 220
ABSTRACT

The foodborne pathogen *Listeria monocytogenes* has a remarkable ability to persist and grow in harsh environments, including the human gastrointestinal tract. This attribute is regulated, at least partly, by the alternative stress-inducible sigma factor, sigma B (σ^B). Although the σ^B regulon is now well defined little is known about the way in which σ^B activity is controlled in *L. monocytogenes*. In this study, the expression of genes that are known to be expressed in a σ^B-dependent manner (*opuCA, lmo2230, lmo2085, sigB*) was measured with qRT-PCR in order to get insights into the conditions that trigger σ^B activation as well as the kinetics. At the transcriptional level the osmotic stress-induced transcription of all four genes was dependent on the extent of the stress applied. During imposition of a sudden osmotic upshock the transcription of all four genes increased rapidly but transiently. To investigate σ^B-dependent expression at the protein level polyclonal antibodies against OpuCA (compatible solute transporter) were developed in chickens and used in Western blotting. OpuCA was expressed in a growth phase dependent manner, with high levels of expression detected in stationary phase. In exponential phase expression levels were low but were induced under conditions of osmotic stress in a manner that was proportional to the magnitude of the stress. Furthermore, based on the transcriptional data, a fluorescent reporter fusion expressing EGFP from strongly σ^B-dependent promoter was developed and introduced to *L. monocytogenes* EGD-e wild-type, ΔsigB and ΔrsbV. A comprehensive analysis of the σ^B activation pattern during growth at 4°C and 37°C and induction in early exponential phase of growth followed by an increased σ^B activity in stationary phase was revealed with flow cytometry at both temperatures. Interestingly, not all of the cells within the wt-egfp population were able to activate σ^B above the highest autofluorescence level detectable by this reporter system. However, σ^B activity can be induced further and proportionally to the extent of stress in exponential phase of growth at 37°C by exposure to osmotic stress. In summary, the data suggest that σ^B activation is heterogeneous within population and can be rapidly modulated in a manner that is proportional to the magnitude of the stress encountered by *L. monocytogenes*. Overall, this study has given new insights into the regulation of a σ^B-driven stress response in *L. monocytogenes* and mechanisms of its adaptability in response to stress.
PUBLICATIONS IN THIS THESIS

The Chapters 2 – 4 of this thesis are based on the following publications and manuscripts:

Chapter 2

Chapter 3
M. Utratna, E. Cosgrave, C. Baustian, R. Ceredig, C. O’Byrne; Development and optimization of an EGFP-based reporter for measuring the general stress response in *Listeria monocytogenes*. Bioengineered; March/April 2012; Volume 3, Issue 2; Pages 93 - 103

Chapter 4
M. Utratna, E. Cosgrave, C. Baustian, R. Ceredig, C. O’Byrne; Effects of growth phase and temperature on $\sigma^B$ activity within a *Listeria monocytogenes* population: Evidence for RsbV-independent activation of $\sigma^B$ at refrigeration temperatures. Submitted to Foodborne Pathogens and Disease; August 2012

Author’s contribution:

- **MU** - designing and performing majority of the experiments, managing reagents and materials, analyzing and interpreting the data, writing the papers, submission and manuscripts reviewing
- **ES** - performing of the transcriptomic study used for the selection of $\sigma^B$-dependent genes; manuscripts reviewing
- **IS** – assisting in development of anti-RsbW, anti-$\sigma^B$ and anti-OpuCA polyclonal antibodies; manuscripts reviewing
- **EC**- performing of the experiments leading to development and optimization of anti-RsbW and anti-$\sigma^B$ polyclonal antibodies; developing of $\Delta rsbV$ mutant; writing the papers and manuscripts reviewing
- **CB** - performing flow cytometry experiments; analyzing and interpreting the flow cytometry data, manuscripts reviewing
- **RC** - analyzing and interpreting the flow cytometry data, manuscripts reviewing
- **COB** - designing and supervising of the all experiments, managing reagents and materials, analyzing and interpreting the data, writing the papers, submission and manuscripts reviewing
LIST OF FIGURES

Fig. 1.1. Taxonomic classification of L. monocytogenes ............................. 16
Fig. 1.2. Progression of L. monocytogenes infection within a human body........ 20
Fig. 1.3. The intracellular life-cycle of Listeria monocytogenes, and the key virulence factors involved................................................................. 23
Fig. 1.4. Listeria pathogenicity island-1 (LIPI-1)........................................... 26
Fig. 1.5. Regulation of prfA transcription..................................................... 28
Fig. 1.6. Regulatory circuits proposed among transcriptional regulators in L. monocytogenes .................................................................................. 36
Fig. 1.7. The proposed model of complex protein-protein interactions regulating σB activity in B. subtilis. ................................................................. 39
Fig. 1.8. Schematic organisation of the OpuC system in L. monocytogenes EGD-e......................................................................................................... 50
Fig. 1.9. Organisation of the sigB operon in L. monocytogenes ....................... 59
Fig. 2.1. Activation of σB by osmotic upshock occurs rapidly and transiently..... 70
Fig. 2.2. Osmotic activation of σB is proportional to the magnitude of the stress... 72
Fig. 2.3. OpuCA expression is induced in proportion to the osmotic stress........ 74
Fig. 2.S1. Induction of opuCA, lmo2230, lmo2085 and sigB transcription in response to osmotic stress is σB-dependent.................................................. 78
Fig. 2.S2. OpuCA expression is σB-dependent................................................ 79
Fig. 3.1. Design of the reporter plasmid pKSV7-P\textsubscript{lmo2230}::egfp containing a fusion of the σB–dependent promoter region of lmo2230 with egfp................................................. 90
Fig. 3.2. Fluorescence of egfp-containing cells of L. monocytogenes EGD-e wild type, ΔsigB and ΔrsbV mutants after transformation with pKSV7-P\textsubscript{lmo2230}::egfp..... 93
Fig. 3.3. EGFP is expressed of L. monocytogenes EGD-e wild type but not in ΔsigB and ΔrsbV backgrounds ........................................................................ 94
Fig. 3.4. σB -dependent expression of EGFP is increased in L. monocytogenes EGD-e wild-type integrant in stationary phase and by osmotic stress............... 96
Fig. 3.5. σB -dependent EGFP expression is induced after osmotic upshock in L. monocytogenes EGD-e wild-type P\textsubscript{lmo2230}::egfp integrant........................................ 97
Fig. 3.6. Heterogeneity of fluorescence within EGFP-expressing population and σB activation proportional to the extent of osmotic stress was revealed with FCM..... 99
Fig. 4.1. Levels of σB and OpuCA at selected points of growth at 37°C and 4°C. 116
Fig. 4.2. Growth of fusion strains at 4°C and 37°C.......................................... 118
Fig. 4.3. $\sigma^B$ is activated in *L. monocytogenes* EGD-e wild-type in growth phase dependent manner at 4°C and 37°C................................................................. 119

Fig. 4.4. Activation of $\sigma^B$-dependent gene expression occurs in the absence of RsbV at 4°C.............................................................................................................. 122

Fig. 4.S1. $P_{lo2230}$ is not activated at 37°C in *L. monocytogenes* EGD-e $\Delta$sigB and $\Delta$rsbV mutants................................................................................................................. 126

Fig. 4.S2. RsbW levels are stable at 4°C and 37°C................................................ 127
LIST OF TABLES

Table 2.1. Primers used in this study (Utratna et al., 2011, Chapter 2) .............. 71
Table 2.1S1. Data supporting Fig. 2.1 and Fig. 2.2 .............................................. 76
Table 3.1. Plasmids and strains used in this study (Utratna et al., 2012, Chapter 3) ............................................................................................................................... 88
Table 3.2. Primers used in this study (Utratna et al., 2012, Chapter 3) .............. 88
Table 4.1. Plasmids and strains used in this study (Utratna et al., 2012, Submitted, Chapter 4) .................................................................................................................. 111
Table 4.2. Primers used in this study (Utratna et al., 2012, Submitted, Chapter 4) ............................................................................................................................ 113
Table 4.3. Antibodies used in this study (Utratna et al., 2012, Submitted, Chapter 4) .......................................................................................................................... 114
LIST OF APPENDICES

Appendix 1. Purification of OpuCA (by Fusion Antibodies) for chicken immunisation and the development of anti-OpuCA polyclonal antibodies............. 167
Appendix 2. The impact of other GI tract-related stresses on the expression of OpuCA.............................................................................................................. 170
Appendix 3. Stability of the OpuCA protein.......................................................... 172
Appendix 4. The activation of $\sigma^B$ is proportional to the magnitude of the pH stress at RNA level........................................................................................................... 173
Appendix 5. Kinetic analysis of gene expression after weak acid upshock........ 175
Appendix 6. Development of the pKSV7-P$_{lmo2230}$::egfp reporter vector........ 178
Appendix 7. Transformation of the pKSV7-P$_{lmo2230}$::egfp reporter vector into L. monocytogenes and chromosomal integration......................................................... 180
Appendix 8. Optimisation of quantification fluorescence microscopy............ 182
Appendix 9. Fixation and filtration of L. monocytogenes fusion strains for quantitative microscopy........................................................................................ 183
Appendix 10. Background subtraction and pixel intensity measurement of digital images, with ImageJ.......................................................... 186
Appendix 11. Particle counting with ImageJ.......................................................... 188
Appendix 12. Calculation of the proportion of fluorescent cells with fluorescence microscopy........................................................................................................... 192
Appendix 13. Optimisation of flow cytometry and defining the gate for $\sigma^B$-dependent expression of egfp.......................................................... 195
Appendix 14. Activation of $\sigma^B$ in L. monocytogenes grown in the range of salt stress investigated at a single cell level ................................................................. 198
Appendix 15. Activation of $\sigma^B$ at the single cell level after treatment with antimicrobials .................................................................................................................. 199
Appendix 16. Development of fusion strains in L. monocytogenes 10403S wild-type, $\Delta$sigB and $\Delta$rsbV' background........................................................................... 203
Appendix 18. M. Utratna, E. Cosgrave, C. Baustian, R. Ceredig, C. O’Byrne; Development and optimization of an EGFP-based reporter for measuring the general
stress response in *Listeria monocytogenes*. Bioengineered; March/April 2012; Volume 3, Issue 2; Pages 93 – 103................................................................. 211

LIST OF ABBREVIATIONS

2-DGE  2-dimensional gel electrophoresis
BHI     brain heart infusion
BHIS    brain heart infusion supplemented with salt
BSA     bovine serum albumin
cDNA    complementary DNA
CFP     cyan fluorescent protein
cml     chloramphenicol
CT      cycle threshold
dH2O    distilled water
ddH2O   double distilled water
DM      defined media
EGFP    enhanced green fluorescent protein
FCM     flow cytometry
g       force of gravity
GFP     green fluorescent protein
IEF     isoelectric focusing
LB      Luria-Bertani
MFI     mean fluorescence intensity
MW      molecular weight
OD      optical density
PCR     polymerase chain reaction
pI      isoelectric point
RNAP    RNA polymerase
rpm     revolutions per minute
RT-PCR  reverse transcriptase PCR
SAM     S-adenosylmethionine
SDS     sodium dodecyl sulphate
SDS-PAGE sodium dodecyl sulphate polyacrylamide gel electrophoresis
SGR     specific growth rate
TEMED   tetramethyl ethylenediamine
wt or WT wild-type
v/v     volume by volume
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>w/v</td>
<td>weight by volume</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION
1.1. LISTERIA MONOCYTOGENES – A SAPROPHYTIC ORGANISM AND OPPORTUNISTIC PATHOGEN

1.1.1. Taxonomy of L. monocytogenes

The bacterium known today as Listeria monocytogenes was described for the first time by E.G.D. Murray and co-workers in 1926, and isolated during an infection in guinea pigs and rabbits (Murray et al., 1926). Because of the increased number of monocytes that were found in the bloodstream of infected animals, the organism was originally named Bacterium monocytogenes. An identical bacterium was isolated independently in the following year, from the liver of gerbils (Pirie, 1927) in South Africa, and named Listerella hepatolytica, in honour of Joseph Lister, a pioneer of antisepic surgery. Its first connection to human disease was suspected quite early (Burn, 1936), but for years, the bacterium was recognized as a veterinary pathogen. The name Listeria monocytogenes has been used since 1940 (Pirie, 1940). An epidemic of L. monocytogenes causing infection of humans was not reported until the late 1970s (Carbonnelle et al., 1979). In 1983, the bacterium was officially recognised as a food-borne pathogen (Schlech et al., 1983). Later, a gene cluster encoding six virulence factors employed in the invasion of eukaryotic cells was described, and paved the way for a better understanding of the pathogenic lifestyle of the organism and the molecular basis of its intracellular parasitism (Vasquez-Boland et al., 2001, Wurtzel et al., 2012).

The genome of L. monocytogenes EGD-e was sequenced in 2001, and revealed a content of 38% G+C and 2,853 protein-coding genes with an average length of 306bp (Glaser et al., 2001). Complete genome sequences for a number of other strains of the genus Listeria are also available (eg. on the GenoList World-Wide Web Server or Listilist). In the phylogenic classification based largely on analyses of the 16S rRNA sequences, rather than on phenotypic data, the genus Listeria (Fig. 1.1) is very close to Brochothrix, and both genera (family Listeriaceae) are positioned between Lactobacillus and Bacillus, belonging to the large, systematic phylum of Firmicutes (Bergey’s Manual of Systematic Bacteriology). Members of the genus Listeria are Gram-positive, small regular rods (0.5 x 2 μm on average) which can form short chains. Listeriae are facultatively anaerobic, non-sporulating and motile at temperatures below 30°C.
Fig. 1.1. Taxonomic classification of L. monocytogenes. A) Global phylogeny of fully sequenced Firmicutes, with arrow indicating members of Listeriae (Ciccarelli et al., 2006). B) Phylogeny of the genus Listeria, containing eight species, including the most recently identified (underlined), L. rocourtiae and L. marthii (Leclercq et al., 2010, Graves et al., 2010). Dotted lines indicate the two most distant species; circles show the two pathogenic species, while three lineages of L. monocytogenes are marked with Roman numerals (Cossart, 2011).

The pathogenic species of the genus Listeria (Fig. 1.1) are: L. monocytogenes, which is the causative agent of foodborne listeriosis in humans, and a source of infection in a number of other host species; and L. ivanovii, which only affects animals, mainly sheep and cattle, with limited infections reported in humans (Cummins et al., 1994, Lessing et al., 1994). L. seeligeri, despite the presence of the homologues of the main virulence gene cluster, has rarely been reported to cause a disease in humans or animals, and is recognised as non-pathogenic (Gouin et al., 1994, Rocourt et al., 1986). A major difference between L. monocytogenes and non-pathogenic L. innocua is the absence of most of the virulence gene cluster, including positive regulatory factor A (PrfA), the master virulence regulator (Chakraborty et al., 2000, Glaser et al., 2001). It has been suggested that the major habitat of ancestral Listeria was soil, and the virulence gene cluster which was needed primarily against soil protozoa evolved at some stage to facilitate the intracellular lifecycle (Chakraborty et al., 2000, Ly and Muller, 1990). Based on genomic comparisons, it has been proposed that progenitor species contained the virulence gene cluster, which was eventually lost by non-pathogenic Listeria (Vasquez-Boland et al., 2001).
Individual isolates of *L. monocytogenes* also exhibit clear differences in terms of their ability to cause disease. Serotyping based on somatic and flagellar antigens discriminates 13 serotypes of *L. monocytogenes*, divided between three lineages at present. Lineage I contains serotypes 1/2b, 3b, 4b, 4d, 4e and 7; lineage II includes serotypes 1/2a, 1/2c, 3a, and 3c; lineage III includes 4a and 4c serotypes (Hain *et al.*, 2012). Only four serotypes (1/2a, 1/2b, 1/2c, 4b) are responsible for most cases of listeriosis in humans, with 1/2a being most commonly found in food and 4b causing disease most often (Glaser *et al.*, 2001).

1.1.2. Incidence of *L. monocytogenes* in nature

*L. monocytogenes* occurs widely in the environment, and can be found in water, soil, plants and decaying vegetation, in the intestinal tracts of animals (e.g., cattle, goats and deer), and their faeces (Gray and Killinger, 1966). Thus, the public health importance of the bacterium is particularly related to its ubiquitous nature and its potential to contaminate a wide variety of raw food, together with re-contamination of food occurring in the processing environment. The bacterium is able to grow slowly even in properly refrigerated food, and it can survive processing based on low pH (pH 2.5) or high salt (up to 3M NaCl) conditions (Gandhi and Chikindas, 2007). *L. monocytogenes* can be found in fresh fruits, vegetables and salads; in milk, dairy products and soft cheeses; in various meats and related products (pates, hot dogs and fermented sausages); in seafood and fish (Rocourt and Bille, 1997, Norton and Braden, 2007).

Due to the life-threatening nature of *L. monocytogenes*, the minimal infective dose of this pathogen for humans is not known, and can only be estimated (Buchanan *et al.*, 1997). Modelling the parameters affecting *L. monocytogenes* growth limits in foods is also extremely complex (Koutsoumanis and Sofos, 2005, Skandamis *et al.*, 2008). A relatively high initial dosage (10⁵-10⁷ CFU/g or 10⁹ CFU/ml) of *L. monocytogenes* in food is required to cause infection in a healthy host (Dalton *et al.*, 1997, McLauchlin *et al.*, 1990). However, a much lower dosage of bacteria (10⁵) can affect individuals with a weakened immune system, including people at extremes of age, patients with HIV, individuals after transplantations and under immunosuppressive therapy (Farber and Peterkin, 1991, McLauchlin *et al.*, 1991a). The cell-mediated immunity is also impaired during pregnancy; thus, pregnant women and their unborn
babies are at a 20-fold higher risk of contracting listeriosis (Mylonakis et al., 2002, Medoff et al., 1971). The most recent EU regulations (EC/1441/2007) state that L. monocytogenes should not be detected in 25 g of foods that can support the growth of the bacterium (EU, 2007). However, the zero-tolerance approach is softened in the case of the food types that do not support the growth over the shelf-life where a low level of listerial contamination ($<10^2$ CFU/g) is allowed.

Despite the statistics showing that the incidence of listeriosis in humans is relatively low, it is associated with a high case-fatality rate of approximately 20–30%, in the face of active antibiotic therapy (Ramaswamy et al., 2007). Most commonly, the disease occurs as sporadic independent cases, with an unclear source of infection. However, outbreaks and contaminated product recalls are still common in developed countries, causing both a serious risk to public health and substantial economical problems (Goulet et al., 1995, Mead et al., 2006, Jacquet et al., 1995, McLauchlin et al., 1991b). The most recent multi-state outbreak of L. monocytogenes-associated infections occurred in the US (http://www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/120811/index.html) between September and December of 2011. The outbreak was caused by the contamination of fresh, whole cantaloupes from one farm in Colorado with two distinct genomic clusters of L. monocytogenes – one containing 1/2a strains and the other containing 1/2b strains (Laksanalamai et al., June 2012). A total of 146 people were infected; 30 deaths and one miscarriage were reported in 28 states. There were several factors identified that have most likely influenced the introduction, growth and spread of L. monocytogenes contamination of cantaloupes, including: low levels of the bacterium in the field where they were grown, inappropriate equipment design in packing facilities and inappropriate post-harvest practices.

Although exposure to L. monocytogenes, due to the ubiquitous nature of the bacterium, cannot be eliminated completely, an increasing knowledge of the physiology and the stress response of this opportunistic pathogen can be incorporated into good agricultural practices, which should be applied routinely by food producers. Then, a much quicker identification of the origins of epidemic food-borne listeriosis can be carried out to reduce additional morbidity and mortality. Furthermore, the risk of infection can be minimised in the future via proper
recommendations to consumers concerning food preparation and storage, especially to people from the high-risk groups (Rebagliati et al., 2009).

1.2. L. MONOCYTOGENES WITHIN THE HOST

1.2.1. Disease manifestations

*L. monocytogenes* enters the human body with contaminated food via the upper gastrointestinal (GI) tract, and migrates through the oesophagus, stomach and duodenum, into the small intestine (Fig. 1.2) (Sleator et al., 2009). A range of other infection sites, from the skin to the conjunctivae of the eye, associated mostly with farmers and the veterinary profession, have been also reported sporadically (Cain and McCann, 1986, Schwartz et al., 1989). A non-invasive form of listeriosis is characteristic for healthy people and it is either asymptomatic or manifests with flu-like symptoms. Due to its ability to enter, survive and multiply within a range of non-phagocytic and phagocytic cells, *L. monocytogenes* can penetrate the intestinal barrier and invade the enterocytes and macrophages of the stroma of the villi, causing gastroenteritis. Because the symptoms are mostly non-specific (fever, diarrhoea, nausea), the non-invasive listeriosis is therefore assumed to be vastly under-diagnosed among immunocompetent people (Dalton et al., 1997).

An invasive form of listeriosis can develop due to the unique ability of *L. monocytogenes* to cross two more barriers of the host: the blood-brain barrier and the fetoplacental barrier (Cossart, 2007). The most common sites away from the original site of infection include the central nervous system, the placenta and the unborn baby. Infections of the central nervous system are manifested as meningitis (or meningoencephalitis), encephalitis or abscesses. Once the pathogen enters the blood and lymphatic system, it infects the spleen and liver. The Kupffer cells of the liver capture the majority of the pathogens from the bloodstream. The accumulation of *L. monocytogenes* is cleared from hepatocytes by a healthy immune system within hours. However, impaired defence mechanisms allow the pathogen to multiply and release the bacteria into the circulatory system. Once *L. monocytogenes* is in the bloodstream, other parts of the body can be invaded via hematogenous spread, leading to septicaemia. In the case of pregnant women, the infection is usually mild,
but it can affect a baby in-utero, leading to death of the foetus, spontaneous abortion, or to listeriosis in the newborn child (Allerberger and Wagner, 2010). Cardiac infections (endocarditis) were also reported (Carvajal and Frederiksen, 1988). Most cases of the invasive form are treatable with prolonged courses of antibiotics – penicillin or ampicillin alone, or in combination with gentamicin. However, due to the bacteriostatic activity of β-lactams against L. monocytogenes, the prognosis of the most serious cases in patients with impaired defence mechanisms is poor (Hof, 2003).

Fig. 1.2. Progression of L. monocytogenes infection within a human body. The bacterium is introduced into the human GI tract (in blue) with contaminated food, and reaches the lower intestine, where it can reside asymptomatically or cause enteritis. L. monocytogenes is able to cross the intestinal barrier (1) and translocate into extraintestinal sites; it reaches the liver and the spleen via the lymphatic system and the bloodstream (in red). The pathogen can further disseminate to establish serious diseases, due to its ability to cross the blood-brain barrier (2) and the foetoplacental barrier (3), in the case of pregnant women. Most common forms of listeriosis, together with the associated symptoms (in brackets), are listed above.
1.2.2. The uptake of *L. monocytogenes* into eukaryotic cells

Two mechanisms of phagocytosis are involved in the uptake of *L. monocytogenes*. The first mechanism is used by the non-phagocytic cells which are not associated with the immune system. At the primary site of infection in the intestinal epithelium, or in hepatocytes in the liver, *L. monocytogenes* induces phagocytosis to enter non-phagocytic host cells via the "zipper" mechanism. This mechanism involves the interaction of a bacterial ligand with a receptor on the host cell (usually a protein involved in cell adhesion). In *L. monocytogenes*, the internalisation process is triggered by at least two proteins – Internalin A (InlA) and InlB – that are expressed on the surface of the bacterium, and were initially identified in non-invasive transposon mutants (Gaillard *et al.*, 1991).

InlA, encoded by *inlA*, is involved in the invasion of epithelial cells, and it is required for crossing the intestinal barrier. InlA interacts with E-cadherin, a host cell adhesion factor expressed at the adherens junction on the basolateral site of the epithelial villi (Adams *et al.*, 1998). *L. monocytogenes* does not actively disrupt the tight junctions, but invades injured epithelial areas or takes advantage of junctional remodelling and mucus secretion to adhere to and attack the epithelium by finding morphologically distinct subsets of intercellular junctions (Pentecost *et al.*, 2010, Pentecost *et al.*, 2006). E-cadherin is also observed in high copy numbers on the basolateral membranes of polarised epithelial cells in the choroid plexus, on the basal and apical plasma membranes of syncytiotrophoblast, and in villous cytotrophoblasts, explaining the tropism of *L. monocytogenes* to the placenta (Lecuit *et al.*, 2004, Shimoyama *et al.*, 1989, Borkowski *et al.*, 1994).

The second internalin, InlB, encoded by *inlB*, promotes entry into a range of different cells, including hepatocytes and epithelial, and several endothelial cell lines (Dramsi *et al.*, 1995, Parida *et al.*, 1998). InlB is loosely attached to the cell wall, and the C-terminus forms an elongated, curved structure able to interact with several ligands (Marino *et al.*, 1999, Jonquieres *et al.*, 2001). The range of InlB receptors include (i) a tyrosine kinase receptor, Met (or hepatocyte growth factor receptor) (Shen *et al.*, 2000); (ii) gC1qR, the complement C1q receptor; and (iii) glycosaminoglycans (GAGs). The interactions induce further signalling events and lead to local rearrangements in the plasma membrane, followed by the engulfment of the bacterial...
cell by an intracellular vacuole. Thus, both internalins mimic normal cell ligands to exploit the junction-forming apparatus and the cytoskeletal rearrangement cascade of the host cell, to internalise the bacterium.

InlA and InlB are not the only internalins identified in the genome of *L. monocytogenes* (Glaser *et al.*, 2001). 22 other internalins were reported, with most of them possessing a homologous cap region but lacking a cell wall anchoring motif. Thus, they are thought to be loosely attached or released extracellularly (Dramsi *et al.*, 1997, Kuhn and Goebel, 2000). InlC was reported to be involved in virulence and in intracellular replication (Engelbrecht *et al.*, 1996, Domann *et al.*, 1997). The role of InlH affects inflammatory response, contributing to survival within host cells (Personnic *et al.*, 2010). Moreover, 30 of the 133 putative surface proteins identified in *L. monocytogenes* EGD-e were not found in non-pathogenic *L. innocua* CLIP11262 suggesting that there might be more surface interactions involved in virulence (Buchrieser *et al.*, 2003).

The second mechanism of phagocytosis is performed by phagocytic cells (e.g. macrophages) of the innate immune system, which are a major line of defence against microbial infections. After engulfment of the bacterium, the phagosome is formed, to be later fused with the lysosome, containing digestive enzymes for a degradation of the ingested material. The final event of this pathway is avoided by *L. monocytogenes*, by escaping through the phagosomal membrane. Thus, invasion of phagocytic cells by *L. monocytogenes* starts with a passive process, and internalins are required for entry into non-phagocytic cells.

### 1.2.3. Intracellular life cycle

To continue the intracellular cycle of *L. monocytogenes*, degradation of the intracellular vacuole is mediated via the expression of a pore-forming cytolysin - listeriolysin O (LLO), encoded by *hly*, which becomes active at the low pH of the phagosome (Fig. 1.3) (Gaillard *et al.*, 1987, Tilney and Portnoy, 1989). The escape process is assisted by two phospholipases: phospholipase C, specific for phosphatidylinositol (PI-PLC), encoded by *plcA* (Camilli *et al.*, 1991), and a broad-range phospholipase C known as phosphatidylcholine phospholipase C (PC-PLC),
encoded by \textit{plcB} \cite{Smith1995}. Additionally, a metalloprotease (Mpl), encoded by \textit{mpl}, is required for PC-PLC maturation.

**Fig. 1.3. The intracellular life-cycle of \textit{Listeria monocytogenes}, and the key virulence factors involved.** Cartoon depicting entry (1), escape from the uptake vacuole (2), cytosolic replication (3), actin-based movement (4) and cell-to-cell spread (5, 6). The cartoon and representative electron micrographs were adapted from (Tilney and Portnoy, 1989).

When the single membrane of the phagosome is lysed, the bacterium is released into the cytoplasm of the host cell \cite{Dramsi2002}. Following the “escape trick”, the bacterium expresses a hexose phosphate transporter (Hpt), which transports glucose-1-phosphate from the host cytoplasm for growth and multiplication \cite{Chico-Calero2002}. Through expression of actin-polymerising protein (ActA), encoded by \textit{actA} \cite{Suarez2001}, \textit{L. monocytogenes} is able to use the host cell’s actin, and reorganise actin filaments to form a polar tail. Asymmetric actin polymerysation propels the bacterial cell in the direction of the host plasma membrane and forms a listeriopod \cite{Sechi1997} directed inside a neighbouring cell. Engulfment of the listeriopod by the adjacent cell generates a double-membraned vacuole-containing bacterial cell. The vacuole is then lysed by the action of the same virulence factors used to escape the first vacuole, and the pathogen can perpetuate the cycle. Thus, \textit{L. monocytogenes} multiplies and spreads from cell to cell without stimulating the humoral response of the host \cite{Tilney1989}. 

23
The global transcriptional profile of *L. monocytogenes* EGD-e during intracellular growth identified 484 genes with altered expression in the murine macrophage (P388D1) cells (Chatterjee *et al*., 2006). The study showed that a total of 41 genes required for intracellular survival are species specific and absent in the genome of non-pathogenic *L. innocua* CLIP11262. A separate study described 541 genes being differentially expressed in *L. monocytogenes* EGD six hours after infection of the epithelial (Caco-2) cell line, a time that reflects cellular replication (Joseph *et al*., 2006). The study suggests that *L. monocytogenes* has a sophisticated strategy for longer survival within the host cell, using alternative carbon and nitrogen compounds and avoiding competition for the host cell’s major sources. Despite different cell lines used in both studies, the gene expression patterns showed an overlap characteristic for adaptation of *L. monocytogenes* to the intracellular environment.

Most recently, small non-coding RNAs (sRNAs) were defined as widespread effectors of post-transcriptional gene regulation of *L. monocytogenes* (reviewed by Mellin and Cossart, 2012). Based on the study comparing putative regulatory RNAs in extracellular and intracellular environments, 29 sRNAs were defined as specifically expressed during intracellular growth of *L. monocytogenes* in macrophages (Mraheil *et al*., 2011). Moreover, these loci are highly conserved in pathogenic *L. monocytogenes* strains, and their involvement in virulence was confirmed by *in vivo* models of infection, opening up a new area of research into regulation of virulence in this pathogen.
1.3. REGULATORS OF GENE EXPRESSION IN L. MONOCYTOGENES

Bacterial pathogenesis and survival rely mostly on the ability to alter gene expression in response to specific environmental stimuli, and to avoid unnecessary energetic costs. Most recently, whole genome-based approaches showed that, in *L. monocytogenes*, gene expression is finely coordinated in a manner that allows the transcription of selected sets of genes specific for different environmental niches and the switching of a saprophytic to a pathogenic lifestyle. Moreover, the bacterium is able to rapidly adapt and change the pattern of the gene’s expression at specific phases of infection within the gastrointestinal tract, in specific host tissues, in various cell types, or even cell compartments.

In the first genome sequence study of *L. monocytogenes*, 201 putative transcriptional regulators were identified (Glaser et al., 2001). In this section, selected well-studied systems that regulate the adaptation of *L. monocytogenes* through altered gene expression are described in detail. PrfA was the first transcriptional regulator identified in the bacterium as responsible for modulating the expression of virulence genes (Leimeister-Wachter et al., 1990). Subsequently, a number of other transcriptional regulators have been reported for *L. monocytogenes*, including: sigma factors of RNA Polymerase (RNAP) (Becker et al., 1998), the heat shock-related negative regulators (HrcA and CtsR) (Hu et al., 2007a, Hu et al., 2007b), a number of two-component regulatory systems (Williams et al., 2005), regulatory RNAs (Mellin and Cossart, 2012) and other regulators. Moreover, regulatory networks showing overlapping interactions and cross-connections between transcriptional regulators of *L. monocytogenes* have been described (Chaturongakul et al., 2011, Toledo-Arana et al., 2009, Milohanic et al., 2003).
1.3.1. Positive regulatory factor A (PrfA)

1.3.1.1. General characteristics and the role of PrfA

The majority of factors that determine the virulence of *L. monocytogenes* identified thus far are regulated by PrfA (Kreft and Vazquez-Boland, 2001). The most important part of the PrfA regulon includes the genes responsible for the intracellular life cycle of the bacterium located within a virulence cluster locus referred to as *Listeria* pathogenicity island-1 (LIPI-1) (Fig. 1.4). PrfA is 27 kDa, and is a site-specific DNA-binding protein. The protein belongs to the cAMP receptor protein (Crp) / Fumarate nitrate reductase regulator (Fnr) family of transcriptional regulators. Similar to most members of the Crp/Fnr family, PrfA is a symmetrical homodimer with the N-terminal domain similar to the cyclic nucleotide-binding domain and the C-terminal domain containing the DNA-binding helix-turn-helix motif. PrfA binds to 5’-TTAACANNTGTAA-3’, a palindromic sequence (‘PrfA-box’) located at the position -41bp of the promoters to which the PrfA–RNA polymerase (RNAP) complex is brought to initiate transcription. The level of PrfA-dependent expression is determined by both the levels of PrfA and its affinity to the target promoter. It is suggested that the affinity is correlated with the perfection of the palindrome sequence, and those with nucleotide mismatches are assumed to have lower degrees of promoter activity.

![Diagram of PrfA regulon]

Fig. 1.4. *Listeria pathogenicity island-1* (LIPI-1) encodes six virulence-associated loci involved in the intracellular life cycle of *L. monocytogenes*: positive regulatory factor A (*prfA*), phospholipase C specific for phosphatidylinositol (*plcA*), listeriolysin O (*hly*), metalloprotease (*mpl*), actin-polymerising protein (*actA*), phosphatidylcholine phospholipase C (*plcB*). Each gene is scaled based on Listilist, and genes pointing to the right are on the positive strand.

Global regulatory capacity of PrfA and the PrfA regulon at the level of the complete genome was described based on a comparison of gene expression profiles between
wild-type *L. monocytogenes* EGD-e and P14 strains with corresponding ΔprfA mutants and P14prfA*, constitutively overexpressing all PrfA-dependent virulence genes (Milohanic et al., 2003). A whole genome array identified a total of 70 genes differentially expressed in the absence of PrfA which are organised in 47 predicted transcriptional units. Three groups of genes with the expression altered by PrfA were identified. Group I consists of the core set of 12 genes proceeded with the PrfA box. The group includes the previously known virulence genes (*prfA-plcA-hlympl-actA-plcB, inlA-inlB, inlC and hpt*), the activation of which by PrfA is increased upon charcoal treatment (Ripio et al., 1996, Geoffroy et al., 1989) and two new genes, *lmo2219* and *lmo0788*. Eight genes negatively regulated by PrfA, and not altered by the presence of charcoal, were placed within group II. Group III consists of the 53 remaining genes, which are PrfA-dependent in BHI and BHI with cellobiose (when PrfA is inhibited). However, the regulatory role of PrfA towards genes from the group III is abolished in BHI when charcoal is added. The promoters of the genes from the latter group do not possess the features of typical PrfA-dependent promoters, but a putative σB promoter was found upstream of more than 60% of genes within this group, suggesting that their transcription might be indirectly regulated by PrfA, possibly through the activity of other regulators (Milohanic et al., 2003).

### 1.3.1.2. Regulation of PrfA

**1.3.1.2.1. Transcriptional regulation of prfA expression**

Expression of *prfA* is controlled at the transcriptional level (Fig. 1.5). The gene can be transcribed either as a part of bicistronic transcript together with *plcA* gene (from *P* _plcA_), or as a monocistronic transcript (from either P1 _prfA_ or P2 _prfA_). Additionally, within P2 _prfA_, two overlapping promoters (σA- and σB-dependent) are found, while P1 _prfA_ is σA-dependent (Nadon et al., 2002, Schwab et al., 2005). Only transcription from *P* _plcA_ is PrfA-dependent, and creates a positive feedback loop leading to the amplification of PrfA expression. In consequence, this autoregulation rapidly increases an expression of the PrfA regulon, including genes within LIPI-1. Transcription from P1 _prfA_ and P2 _prfA_ is required for escaping from the vacuole (Freitag and Portnoy, 1994), while P _plcA_ is used for the spread to neighbouring cells (Camilli et al., 1993).
Fig. 1.5. Regulation of prfA transcription. Transcription of prfA occurs from the known promoters indicated by ‘P’. The illustration is not to scale.

1.3.1.2.2. Translational regulation of prfA expression

Expression of PrfA is temperature-dependent and tightly controlled by an RNA thermosensor (See section 1.3.4). The thermosensor is located in a large 116-nucleotide 5’UTR (untranslated region, with a modulatory role starting at the promoter and ending with the start codon) upstream of the coding part of prfA mRNA. The thermosensor forms a secondary structure (RNA hairpin), and prevents translation of prfA by obstructing access to its Shine-Dalgarno sequence. The hairpin is stable at low temperatures (below 30°C), and it melts at higher temperatures, which is consistent with the requirement of higher levels of PrfA within the mammalian host and upregulation of virulence-associated genes at 37°C (Johansson et al., 2002). The thermosensor mechanism was described for the P1<sub>prfA</sub> only. Separate studies have shown that, at 25°C and 30°C, some virulence genes can still be expressed (Mansfield et al., 2003), suggesting an existence of alternative mechanism under low levels of PrfA.

Furthermore, a recent study discovered the involvement of S-adenosylmethionine (SAM) sensing riboswitches in prfA regulation (Loh et al., 2009). SAM riboswitch element A (SreA) and B (SreB) bind to the 5’UTR of the prfA, and prevent its expression, with a tertiary structure formed. SreA was shown to interact with the region 80bp upstream of the prfA Shine-Dalgarno sequence. The transcription of sreA is PrfA-dependent; thus, it creates a negative feedback loop – when levels of SreA increase, prfA expression is reduced. The finding reports the first SAM riboswitch as acting against distally located target RNA (trans acting), in contrast to
previously described riboswitches regulating the expression of downstream genes (*cis* acting).

1.3.1.2.3. **Post-translational regulation of PrfA.**

Once *prfA* is fully expressed, it can be present in either an active or inactive state (Renzoni *et al*., 1997). Structural studies of *prfA* mutants suggested that PrfA requires conformational changes for its activity (Eiting *et al*., 2005, Vega *et al*., 2004). Activation of PrfA by binding the low molecular weight ligand was proposed based on the homology of PrfA to Crp protein, triggered to the active state by association with cAMP (Velge *et al*., 2007). A variant of PrfA, with a Gly-145Ser substitution that has constitutive activity (PrfA*), was described (Vega *et al*., 2004). The mutation is similar to that observed to a cAMP-independent variant of Crp protein which also poses constitutive activity, suggesting that PrfA* shares with Crp an analogous mechanism of cofactor mediated allosteric shift. To date, neither this ligand nor its origin were identified, but the data gained provide evidence of its existence.

A number of studies using various media compositions described metabolic pathways influencing PrfA activity. The PrfA regulon is induced under starvation (minimal essential medium), or in nutrient-rich conditions (BHI medium) supplemented with active charcoal (Ripio *et al*., 1996, Bohne *et al*., 1994). When the *L. monocytogenes* overexpressing PrfA (PrfA*) were grown in a glucose-containing minimal medium, the excess PrfA* reduced the rate of glucose uptake, which is explained by several down-regulated genes being involved in carbon and nitrogen metabolism (Mertins *et al*., 2007, Marr *et al*., 2006). In the case of *L. monocytogenes* wild-type grown in the presence of glucose, maltose or cellobiose, the activity of PrfA is repressed, but the levels of PrfA are unaffected, showing that the virulence gene expression is regulated by a carbon source (Milenbachs *et al*., 1997).

The uptake of non-phosphorylated sugars is controlled by the phosphoenolpyruvate phosphotransferase system (PTS). Expression of the PrfA regulon is correlated with the phosphorylation status of the PTS permeases (Stoll *et al*., 2008). The permeases receive phosphate groups from enzyme II domain A (EIIA) when non-phosphorylated sugars are obtained. This causes an accumulation of non-
phosphorylated EIIA, inhibiting PrfA activity. In contrast, phosphorylated sugars (glucose-1-phosphate, glucose-6-phosphate), which are abundant in mammalian cells, do not repress PrfA activity. In this case, EIIA is phosphorylated, and PrfA can be active. Within the host, *L. monocytogenes* utilises a non-PTS-dependent uptake system, such as of the hexose phosphate transporter (hpt), which is indeed controlled by PrfA (Chico-Calero *et al.*, 2002). The findings are in line with the requirement of virulence gene expression during intracellular life. In summary, PrfA, the main regulator of virulence gene expression in *L. monocytogenes*, is tightly controlled at the levels of transcription, translation and post-translational modification of its activity. Virulence-associated genes can be expressed at a very precise moment of infection, and are mostly down-regulated outside the host to allow expression of the set of genes required for non-host niches.

### 1.3.2. Sigma factors of RNA Polymerase in *L. monocytogenes*

Bacterial genomes encode multiple interchangeable sigma (σ) factors of RNA polymerase (RNAP). In general, a sigma factor is temporarily bound to the inactive core (subunits α₂ββ’ω) of RNAP to form a holoenzyme, the active form essential for transcription initiation (Borukhov and Nudler, 2003). The RNAP holoenzyme participates in the recognition of a distinct set of promoters with a strictly defined sequence. Thus, it is involved only in the initiation of transcription, and dissociates from the RNAP complex at the early elongation stage. A set of genes expressed as a result of the association of a given σ factor with the RNAP core enzyme defines the regulon of that σ factor. The ability to target RNA polymerase to a specific promoter sequence by a specific sigma factor allows rapid and powerful transcriptional reprogramming in any given conditions encountered, and is sensed by the bacterial cell, determining its survival.

Five RNAP sigma factors are encoded in the genome of the majority of *L. monocytogenes* lineages (Glaser *et al.*, 2001). The primary sigma factor Sigma A (σ^A_\text{}) is encoded by *rpoD* (*lmo1454*) and recognised as a housekeeping sigma factor responsible for transcription in the growing cell. It belongs to the σ^70_\text{ family of sigma factors, and is characterised by a high similarity to the principal sigma factor σ^A_\text{ of B.*}
subtilis (Metzger et al., 1994). The group of four remaining sigma factors described as secondary or alternative sigma factors includes Sigma B (σ^B), Sigma H (σ^H), Sigma 54 (σ^54) and Sigma C (σ^C).

σ^H (encoded by sigH or lmo0243) was suggested to be involved in protection against changing pH, both acidic (Phan-Thanh and Mahouin, 1999) and alkaline (Rea et al., 2004). Growth of ΔsigH was also affected in a minimal medium (Rea et al., 2004). Whole genome microarray analyses of L. monocytogenes 10403S ΔsigH and its isogenic wild-type grown up to stationary phase identified 97 genes as positively regulated by σ^H (Chaturongakul et al., 2011). Putative σ^H-dependent promoter was found upstream of 56 genes.

σ^L, also known as σ^54, is encoded by sigL, rpoN or lmo2461. Its role in the stress response was also suggested by an involvement in resistance to bacteriocin mesentericin Y105 (Robichon et al., 1997, Dalet et al., 2001). ΔsigL demonstrated impaired growth in the presence of salt, under lactic acid stress and at low temperatures (Raimann et al., 2009). Increased transcript levels of sigL were observed at 10 °C (Liu et al., 2002), and ΔsigL was reported to be defective for utilising carnitine (Okada et al., 2006). Whole-genome microarray and proteome analyses of L. monocytogenes EGD-e sigL mutants revealed the altered expression of 77 genes, with 20 of them being positively regulated and involved in regulation of carbohydrate and amino acid metabolism (Arous et al., 2004). The most recent study of L. monocytogenes 10403S sigL mutants reported 31 genes being positively regulated by σ^L in the stationary phase, with 16 of them possessing σ^L promoter (Chaturongakul et al., 2011). A very little overlap between a list of σ^L-dependent genes, arising from the two transcriptomic studies, was explained by different experimental conditions and strains used.

σ^C is an extracytoplasmic function (ECF) sigma factor encoded by sigC, sigV or lmo0423. It is present only in strains of L. monocytogenes that belong to lineage II, and was reported to be activated by heat stress (Zhang et al., 2003). Transcriptomic study aiming to identify the σ^C regulon in the stationary phase did not find any σ^C-dependent genes under selected criteria (Chaturongakul et al., 2011). Further microarray experiments after 10 min exposure to 50°C, and in the strain
overexpressing sigC, identified only three members of the $\sigma^C$ regulon (lmo1854, lmo2186-lmo2185). Among 11 genes with $\sigma^C$ promoter identified upstream with HMM, only one gene (lmo0422) located within the same operon as sigC was confirmed to be $\sigma^C$-dependent by qRT-PCR (Chaturongakul et al., 2011).

$\sigma^B$ (encoded by sigB or lmo0895) positively regulates the transcription of class II stress response genes. It was identified based on the homology of sigB from non-pathogenic Bacillus subtilis, and was first shown to be activated in response to osmotic (Becker et al., 1998) and acid stress (Wiedmann et al., 1998). The wider role of $\sigma^B$ in the general stress response and virulence of L. monocytogenes will be discussed later, in great detail, as the research presented in this study is focused on the regulation of $\sigma^B$ activity in this pathogen.

1.3.3. Other transcriptional regulators
Recently, a new virulence regulator, VirR, has been identified as the second main regulator of virulence genes in L. monocytogenes, controlling mainly genes involved in the modification of bacterial surface components implicated in cell invasion (Mandin et al., 2005). VirR is a DNA-binding regulator, and part of a two-component system, along with VirS. Both proteins are encoded in a five-gene operon, with virS located downstream of virR and separated by three other genes. However, the putative sensor kinase VirS is not necessary for virulence, and does not prevent activation of VirR, suggesting the possibility of an interaction with other sensory histidine kinases. VirR positively regulates five genes of its own operon, and 12 other genes organised in three operons and four single units. Thirteen of these genes were shown to be upregulated in L. monocytogenes isolated from mice spleens (Camejo et al., 2009). The in vivo upregulated genes include the virR and virS genes themselves, the dlt operon necessary for D-alanylation of lipoteichoic acid and mprF, required for lysinylation of phospholipids in the cytoplasmic membrane. The changes reduce the negative charge of the bacterial surface and confer resistance to cationic antimicrobial peptides (CAMPs) (Thedieck et al., 2006). Modifications of lipoteichoic acid might also be used by L. monocytogenes to avoid the host’s innate immune response (Takeda and Akira, 2005).
In addition, the genome of *L. monocytogenes* contains 15 additional predicted two-component systems (Williams *et al.*, 2005). DegU is a response regulator in the absence of the sensor kinase DegS, implicated in the regulation of some *Listeria* secreted proteins (*gap, tsf, sod, lmo0644*) and was upregulated in infected mouse spleens (Camejo *et al.*, 2009). Similarly, OhrR, a transcriptional regulator controlling OhrA, which is a hydroxyperoxidase implicated in intracellular survival, was also upregulated *in vivo* (Gueriri *et al.*, 2008).

Analysis of the contribution of HrcA protein (heat regulation at CIRCE [controlling inverted-repeat chaperone expression element]) to the stress response and regulatory networks of *L. monocytogenes* revealed that this transcriptional regulator has a negative influence at the expression of the class I stress response genes (Hu *et al.*, 2007a). The HrcA regulon consists of 61 genes either directly or indirectly regulated by HrcA. 25 of them are negatively regulated by HrcA, while 36 genes show lower transcript levels in the Δ*hrcA*. A deletion of the *hrcA* gene has limited effects on invasion efficiency, or heat and acid stress resistance. CtsR (class three stress repressor) is a transcriptional regulator repressing at least 64 genes in *L. monocytogenes*, including a direct repression of at least 10 genes (Nair *et al.*, 2000). The regulatory role of CtsR in *L. monocytogenes* was revealed by phenotypic characterisation of Δ*ctsR* mutants under a variety of stress conditions, including heat, acid, oxidative, and high-pressure stress (Joerger *et al.*, 2006, Karatzas *et al.*, 2003).

### 1.3.4. Regulatory RNAs

The first report of regulatory RNAs in *L. monocytogenes* identified with the cloning approach (Barry *et al.*, 1999) was followed by a description of 20 further reported using northern blotting and co-immunoprecipitation with a mRNA-binding chaperone Hfq (Christiansen *et al.*, 2006, Mandin *et al.*, 2007, Nielsen *et al.*, 2008). Recently developed techniques (e.g. genomic tilling arrays or next-generation DNA sequencing for high-throughput RNA analysis through cDNA sequencing [RNA-Seq]) allowed an identification of a total of 180 regulatory RNAs, mostly non-coding, in the genome of *L. monocytogenes* (Toledo-Arana *et al.*, 2009, Mraheil *et al.*, 2011, Oliver *et al.*, 2010). Subsequently, an unexpected abundance of regulatory RNAs within the genome, together with the level of complexity and an association of
their role in modulation of virulence of *L. monocytogenes*, opened a new field of research into the regulation of the pathogenic lifestyle in this organism.

RNA/mRNA interactions alter gene expression via the destabilisation of mRNA, and in general, occur through three different classes of regulatory RNAs (Gripenland *et al.*, 2010). The first class consists of *trans*-acting, small, non-coding RNAs (sRNAs) encoded in intergenic regions separate from a target gene. In *L. monocytogenes*, 101 sRNAs were identified thus far, measuring usually 50-150 nucleotides in length (Mraheil *et al.*, 2011, Toledo-Arana *et al.*, 2009, Oliver *et al.*, 2010). Their function is mostly unknown; however, 29 of the sRNAs were shown to be exclusive for intracellular conditions (Mraheil *et al.*, 2011). Moreover, some sRNAs (*rliB, rli33-1, rli38* and *rli50*) absent in nonpathogenic *L. innocua*, were proposed to be involved in virulence, based on the decreased survival rates of selected knockouts in murine and invertebrate infection models (Toledo-Arana *et al.*, 2009).

Anti-sense RNAs (asRNAs), encoded on the strand of DNA complementary to the target transcript, are the second class of regulatory RNAs. More than 50 of asRNAs were identified in the genome of *L. monocytogenes* (Toledo-Arana *et al.*, 2009). Based on the localisation, they can be either (i) covering one or more ORFs without overlap to the flanking regions, or (ii) 3’ or 5’UTRs of mRNA overlapping with the mRNA encoded on the opposite strand. The asRNA named *anti2095* covers *lmo2095*, but also *lmo2096* and *lmo2097*, and is similarly observed for *anti2325* or *anti2394*. The second group is represented by 13 overlapping 3’UTRs and 7 overlapping 5’UTRs, including the well-described transcriptional repressor of flagellum genes – *mogR* (Toledo-Arana *et al.*, 2009, Mraheil *et al.*, 2011).

The third group of regulatory RNAs consists of *cis*-regulatory 5’UTRs acting as sensors of temperature and metabolites (riboswitches). Regulation of PrfA by 5’UTR (section 1.3.1.1) is a classic example of a thermosensor in *L. monocytogenes* (Johansson *et al.*, 2002). A number of riboswitches that possess an RNA region (aptamer) specific to a metabolite which alters the structure, resulting in the termination of the transcription of the downstream gene, were reported for *L. monocytogenes* (Loh *et al.*, 2009, Mansjo and Johansson, 2011, Toledo-Arana *et al.*, 2009, Mraheil *et al.*, 2011). The lysine ryboswitch, LysRS, prevents transcription of
the lysine transporter (*lmo0798*) located downstream in the presence of high concentration of lysine. Interestingly, LysRS can also act as a 3’UTR and dual 3’UTR, and 5’UTR functions have been also reported for other riboswitches sensing cobalamin, T-box, M-box and S- SAM (Toledo-Arana *et al*., 2009). The *trans*-acting mode of action for SAM riboswitches has also been reported and investigated in *prfA* regulation (section). The flavin mononucleotide (FMR) riboswitch, involved in the regulation of riboflavin, transporting genes directly controlling bacterial growth, has been also described (Mansjo and Johansson, 2011).

### 1.3.5. Regulatory networks

The regulons of each transcriptional regulator described thus far in *L. monocytogenes* are not completely independent, but rather tightly interconnected in the gene expression network forced by the requirement of adaptation. The most strongly supported example of a direct link between important transcriptional regulators in *L. monocytogenes* is described for PrfA and \( \sigma^B \), suggesting an overlap between expression of virulence and stress response genes. One of the three promoters upstream of *prfA* is regulated by \( \sigma^B \) (Fig. 1.5 in section 1.3.1.2.1) (Nadon *et al*., 2002, Schwab *et al*., 2005), and the group of genes reported as PrfA-dependent have putative \( \sigma^B \) promoters (Milohanic *et al*., 2003).

An extensive transcriptomic study has recently described gene expression profiles of *L. monocytogenes* in response to number of conditions that are encountered during the infection of the host (Toledo-Arana *et al*., 2009). Transcription profiling of *L. monocytogenes* in the intestine found the altered expression of 1,206 genes. Many of upregulated genes were predicted to be involved in stress adaptation, and 20% were shown to be under control of the stress response sigma factor, \( \sigma^B \). Analysis of the transcript levels of *L. monocytogenes* in blood showed an overlapping region between two regulons. However, a decreased role of \( \sigma^B \)-dependent adaptation and overexpression of genes regulated by PrfA observed in blood suggests a fine-tuning of gene expression in response to changing environmental conditions, characteristic of different stages of infection. (Toledo-Arana *et al*., 2009).

\( \sigma^B \) is also involved in regulation of the stress-inducible transcription of Hfq, the RNA-binding protein, and an important regulatory factor in the modulation of the
function of non-coding RNAs participating in a variety of physiological processes, including virulence-associated genes in *L. monocytogenes* (Christiansen *et al.*, 2004). Transcriptional networks were reported for $\sigma^B$ and two repressors, CtsR and HrcA, which negatively regulate heat shock genes (e.g., *clpP*, *clpC*, *dnaK*, and *groES*), and suggests that *L. monocytogenes* coordinates the response to various environmental stresses (Hu *et al.*, 2007a, Hu *et al.*, 2007b).

A whole-genome microarray analysis was utilised to identify any overlap among regulons of transcriptional regulators, including all four alternative sigma factors of *L. monocytogenes* ($\sigma^B$, $\sigma^C$, $\sigma^H$, and $\sigma^I$), the virulence gene regulator PrfA and the heat shock-related negative regulators CtsR and HrcA (Chaturongakul *et al.*, 2011). $\sigma^B$ was shown to co-regulate 176 genes under the conditions tested, and was proposed to be a central regulator that connects many other regulons in the network (Fig. 1.6). 188 genes were shown to be controlled by more than one regulator, while 25 genes were identified as being co-regulated by three or more regulators. $\sigma^B$ and $\sigma^H$ coregulate 92 genes, and 39 of these are positively regulated by both. $\sigma^H$ is involved in the regulation of 31 $\sigma^B$-dependent genes (10 positively).

*Fig. 1.6. Regulatory circuits proposed among transcriptional regulators in *L. monocytogenes*. The model was reprinted from (Chaturongakul *et al.*, 2011).*
Moreover, the study emphasized a compensation phenomenon – the loss of major regulators (sigH, sigL, and sigC) in *L. monocytogenes* 10403S did not result in any phenotypic consequences under conditions tested, suggesting that regulatory networks help bacteria in adapting to changes in genome components. The same non-affected phenotype was reported before for ΔsigB strain grown under selected experimental conditions, suggesting that the biological functions of this important regulator can be taken over by other regulators (Hain *et al.*, 2008, Abram *et al.*, 2008a, Chaturongakul and Boor, 2004). The interaction of network components are also affected by strong competition between the individual sigma factors for interaction with RNAP. Thus, the number of molecules of each sigma factor in a bacterial cell, and the pool existing in an active form, determine the relative level of gene expression mediated by each sigma factor, and are crucial for transcriptional remodelling when certain conditions are encountered.
1.4. ALTERNATIVE SIGMA FACTOR SIGMA B IN B. SUBTILIS

1.4.1. Discovery and regulation of σB in B. subtilis

The ability of bacteria to sense and respond to changes in their environment depends partly on transcriptomic reprogramming; due to the availability of multiple RNAP sigma (σ) factors (section 1.3.2). σB sigma factor (initially named σ37, based on a molecular mass of 37 kDa) was identified in an extract from early sporulating B. subtilis (Haldenwang and Losick, 1979). It was the first σ subunit able to transcribe template genes spoVG and ctc in vitro, in addition to previously reported σA. σB protein was isolated from a RNAP complex by dissociation with 6 M urea and fractionation with chromatography on phosphocellulose. The isolated factor was able to reconstitute transcription when added back to the purified core of RNAP (Haldenwang and Losick, 1980). The promoter sequence unique for the σB subunit was described soon after for the ctc (catabolite controlled) gene, and its conserved sequence was later routinely used for reporting σB-dependent transcription in B. subtilis (Moran et al., 1981).

The follow-up genetic studies by two independent laboratories cloned the gene encoding the σB subunit, and identified its location on the B. subtilis genome using a deduced oligonucleotide sequence (Binnie et al., 1986) or anti-σB antibodies-based approach (Duncan et al., 1987). Moreover, both studies suggested that disruption of the sigB gene did not abolish growth or the sporulation ability of B. subtilis. Analysis of the sequence surrounding the sigB gene revealed that it is the third gene within a four gene operon orfV-orfW-sigB-orfX, and σB itself is involved in its transcription (Kalman et al., 1990). A high similarity between the genetic organisation of the sigB operon and that of the B. subtilis spoIIA (sigF) operon was also observed. Thus, the two upstream adjacent genes were proposed to encode Rsb proteins (regulators of sigma b).

The regulatory roles of the genes lying within the sigB operon (re-named as rsbV-rsbW-sigB-rsbX) and co-expressed in a σB-dependent manner from the promoter located upstream from rsbV, were determined by a series of mutations within the operon. Subsequently, the role of RsbV as a positive regulator of σB activity, together with a negative role for RsbW and RsbX, were described (Boylan et al., 1992,
Benson and Haldenwang, 1992). A partner-switching mechanism assuming RsbV interactions with either RsbW or $\sigma^B$ was proposed based on coprecipitation and Western blot experiments (Benson and Haldenwang, 1993a). RsbW was shown to be an anti-sigma factor sequestering $\sigma^B$ and preventing its interactions with the core of RNAP (Fig. 1.7B). The key role of the phosphorylation status of RsbV in the partner switching mechanism was revealed with isoelectric focusing and Western blotting (Dufour and Haldenwang, 1994). In a dephosphorylated form, RsbV is able to act as an anti-anti-sigma factor by sequestering RsbW. This interaction allows $\sigma^B$ to be released from the RsbW-$\sigma^B$ complex, and to participate in the transcription of $\sigma^B$-dependent genes.

Fig. 1.7. The proposed model of complex protein-protein interactions regulating $\sigma^B$ activity in *B. subtilis*. A large protein complex called “stressosome” is composed of multiple copies of RsbR, RsbR paralogues (RsbR$_P$), RsbS and RsbT (A). When a stress signal is perceived, RsbR and RsbS become phosphorylated. This causes a release of RsbT from the stressosome. RsbT activates RsbU, which can dephosphorylate RsbV. Once RsbV is dephosphorylated (B), it can sequester RsbW, releasing $\sigma^B$ for participation in transcription. Energy stress is sensed by a separate RsbQP pathway (C). Both RsbU and RsbP can shift the equilibrium from phosphorylated to dephosphorylated RsbV, and consequently, trigger stress gene activation.
Four additional genes (*rsbR, rsbS, rsbT* and *rsbU*) involved in the regulatory cascade of ΣB in *B. subtilis* were identified directly upstream from *rsbV*, and they were shown to be co-transcribed with the downstream four-gene operon in a ΣB-independent manner (Wise and Price, 1995). The interactions between RsbR, RsbS, RsbT and RsbU were further investigated, and led to the description of a complex model involving protein-protein interaction activating ΣB in response to stress (Akbar *et al.*, 1997, Kang *et al.*, 1996). An electron microscopy approach revealed that RsbR and RsbS form a large multisubunit complex (Chen *et al.*, 2003), and further biochemical data demonstrated that the complex traps RsbT in the absence of stress, preventing the latter from activating RsbU (Fig. 1.7A) (Delumeau *et al.*, 2006).

A cryo–electron microscopy reconstruction of the RsbR-RsbS-RsbT complex has been published recently, and renamed a ‘stressosome’ due to its proposed role in sensing stress by various domains in RsbR paralogues (Marles-Wright and Lewis, 2008). C-termini of RsbR share high sequence similarity due to the presence of the STAS (Sulphate Transport Anti Sigma) domain, which probably plays a role in the assembly of the stressosome. N-termini of the RsbR paralogues (RsbRP) share a low level of similarity between the paralogues, and form protrusions out of the core of the stressosome. Thus, N-terminal parts of RsbRP were proposed to create a range of receptors of the stressosome responsible for stress sensing. One of RsbR paralogues (YtvA) has been shown to sense blue light with its light-oxygen-voltage (LOV) domain (Gaidenko *et al.*, 2006), suggesting that the stressosome might also be involved in integrating a range of environmental signals into the pathway for activating ΣB. RsbR and RsbS are also targets of phosphorylation by the protein kinase RsbT, which is in turn required for the activation of RsbT and the transmission of the stress signal into the downstream cascade (Gaidenko *et al.*, 1999). Once the stress signal is perceived by the stressosome, RsbT is released from the complex, and is able to activate the RsbU phosphatase. RsbU then causes dephosphorylation of RsbV, which disrupts the ΣB-RsbW complex, releasing active ΣB (Delumeau *et al.*, 2006).

Early studies of the regulatory cascade reported that the activation of ΣB is abolished in the ΔrsbU strain, in response to environmental stress (salt stress, heat shock or ethanol), but not under energy stress (resulting from carbon, phosphate or oxygen
starvation), suggesting the existence of an alternative mechanism of $\sigma^B$ activation (Voelker et al., 1995). The separate energy pathway of $\sigma^B$ induction is regulated by two proteins encoded distally from $\text{sigB}$ operon and lying within $\text{rsbQP}$ operon (Brody et al., 2001). RsbP is another RsbV-specific phosphatase (Fig. 1.7A), but its N-terminal region contains a PAS sensory domain able to detect changes in energy levels. RsbP is expressed in an inactive form, and needs the $\alpha/\beta$ hydrolase activity of RsbQ, which binds unidentified hydrophobic molecules to a potential cofactor or to RsbP itself (Kaneko et al., 2005). However, the initial stress-sensing mechanism remains to be elucidated.

1.4.2. Tracking $\sigma^B$ activity in $B.\ subtilis$

In addition to elucidating components of the $\sigma^B$ activation cascade in $B.\ subtilis$, numerous studies addressed questions of when, or under what conditions, $\sigma^B$ is active in the cell. The approaches used to track $\sigma^B$ activity in response to stress ranged from investigating the expression of $\sigma^B$-dependent genes and $\text{sigB}$ itself (at RNA and protein level), to measuring the expression of products expressed from $\sigma^B$-dependent promoters in reporter fusions. A wide spectrum of experimental conditions tested over two decades allowed the creation of a comprehensive picture, and has led to better understanding of the modulation of $\sigma^B$ activity and general stress response in $B.\ subtilis$.

1.4.2.1. Identification of genes reporting $\sigma^B$ activity in $B.\ subtilis$

$s\text{poVG}$, the early sporulation gene, was first shown to be transcribed by purified $\sigma^B$ in the absence of $\sigma^A$ in vitro (Haldenwang and Losick, 1979). In the fragment of DNA cloned to investigate the role of $\text{spoVG}$ in sporulation, the $\text{ctc}$ gene (initially considered as $\text{spoVC}$) was also included, and was demonstrated to be transcribed in a $\sigma^B$-dependent manner both in vitro and in vivo. In contrast, the role of $\sigma^B$ in the expression of $\text{spoVG}$ was not confirmed in vitro (Wise and Price, 1995). Moreover, $\text{ctc}$ was found to be a distinct gene from the $\text{spoV}$ operon, and was shown not to be required for sporulation (Ollington et al., 1981). Since $\text{ctc}$ was known to be transcribed by RNAP-$\sigma^B$, its promoter was fused to the reporter genes $\text{cat}$, $\text{xylE}$ and
lacZ (Igo and Losick, 1986). Despite identification of another $\sigma^B$-independent promoter contributing to the transcription of ctc (Ollington and Losick, 1981), the expression of ctc-lacZ was not detectable in a sigB mutant (Igo et al., 1987). The reporter fusions of ctc were widely used to investigate conditions that $\sigma^B$ is active (or inactive) at (Benson and Haldenwang, 1993b, Boylan et al., 1993a, Igo and Losick, 1986, Volker et al., 1994), and to identify mutations that enhance or inhibit ctc expression (Igo et al., 1987, Ray et al., 1985). The most recent study of the ctc locus revealed that it encodes a 50S ribosomal protein from the L25 family. Ctc is induced under stress conditions and required for accurate translation (Schmalisch et al., 2002).

Experiments with transcriptional fusions to all four genes of the sigB operon revealed that the expression of the reporter protein is abolished in sigB mutants, and concluded that the sigB gene itself is positively regulated by $\sigma^B$ (Kalman et al., 1990). Primer extension analyses, together with nuclease mapping, identified transcripts starting upstream from the promoter (P$_{sigB}$) located upstream from rsbV-rsbW-sigB-rsbX, with the sequence identical to ctc promoter. The existence of an additional $\sigma^B$-independent promoter taking part in sigB expression was first suggested more than 1,100 bp upstream from the sigB gene (Kalman et al., 1990). Due to sigB autoregulation, both P$_{sigB}$ (in lacZ or fluorescent fusions) and the expression levels of four genes lying within downstream parts of the sigB operon were used to report $\sigma^B$-dependent expression in B. subtilis. However, early studies suggested that $\sigma^B$-dependent fusions with the promoter from sigB operon do not respond in an identical manner, and that the degree of induction of the sigB is lower than the other reporter genes (Benson and Haldenwang, 1992). Once posttranslational regulation was proposed for $\sigma^B$, the levels of $\sigma^B$ or Rsb proteins assessed with monoclonal antibodies were not considered as an indication of $\sigma^B$ activity (Benson and Haldenwang, 1993b). Additionally, $\sigma^A$ promoter was later localised upstream of rsbR, proving that $\sigma^B$-independent expression must be taken into consideration during experimental design (Wise and Price, 1995).

Another gene with a promoter controlled by $\sigma^B$, and highly similar to the reported consensus sequence, was identified among glucose starvation inducible units, and was named gsiB (Volker et al., 1994, Mueller et al., 1992). The gene encodes the
hydrophilic protein of 14 kDa, and is induced by a number of environmental stresses and starvation (Mueller et al., 1992), conditions characteristic of the activation of other σ^B-dependent genes (Maul et al., 1995, Voelker et al., 1995). Despite the reports of the role of σ^A in the transcription of gsiB, its transcription by the RNAP-σ^A complex was not detected in vivo (Volker et al., 1994). Thus, both expression from the gsiB promoter and GsiB levels were used to report σ^B activity (Mostertz and Hecker, 2003, Kovacs et al., 1998, Brigulla et al., 2003, Holtmann et al., 2004).

Screening of the Tn917 lacZ library was carried out in order to search for maximal expression that requires an intact σ^B, and identified csbA as another σ^B-dependent gene with an induction pattern similar to ctc (Boylan et al., 1991). It encodes an 8 kDa putative membrane protein that is basic and hydrophobic. csbA is transcribed from σ^B promoter, but a weaker promoter similar to σ^A is located directly upstream of the gene. The genetic approach described above was repeated for the rsbX mutant, displaying increased σ^B-dependence in the absence of a negative regulator of σ^B. Eleven additional csb genes (controlled by sigma B) were isolated, and were shown to be involved in the response to the stationary phase stress (Boylan et al., 1993b). One of the genes identified (csb42) encodes a 32 kDa UDP-glucose pyrophosphohydrolase, involved in cell wall synthesis, and was renamed gtaB (Varon et al., 1993). Its assigned role first suggested that σ^B might be involved in responses to other kinds of environmental stresses, in addition to stationary phase stress. Levels of expression of csbA served as an indication of σ^B-dependent gene expression under environmental stresses (Benson and Haldenwang, 1993c, Boylan et al., 1992), or after rifampin treatment (Bandow et al., 2002). csbA fusions were also utilised to prove that selected mutations in the σ^B regulatory proteins affected σ^B activity in general, and were not restricted to ctc (Boylan et al., 1992).

In summary, a number of strongly σ^B-dependent genes were identified in the pregenomic era, to study σ^B activity in B. subtilis. Interestingly, all of the reporter genes were later confirmed to be members of σ^B regulon, by global proteomic (Bernhardt et al., 1999) and transcriptomic approaches (Price et al., 2001, Petersohn et al., 2001). Despite a basal level of σ^B-independent expression of some reporter genes (mostly due to a weak σ^A promoter), their stress induction was attributed to σ^B. Expression of those genes was measured via a wide range of methodologies,
including enzymatic and fluorescent fusion reporters, RNA-based approaches (Northern Blotting, qRT-PCR, primer extension analysis), proteomics and antibody-based analyses to elucidate the role of $\sigma^B$ in the stress response of *B. subtilis* and understand modulation of that response.

### 1.4.2.2. Modulation of $\sigma^B$ activity in *B. subtilis* in response to stress

The physiology of adaptation and global understanding of gene regulation in response to environmental stress and starvation in *B. subtilis* has been investigated for almost three decades. Early studies aimed at visualising global changes in the gene expression patterns using proteomics, and identified large group of nonspecific stress proteins induced by glucose, phosphate or oxygen starvation, as well as salt, acid, ethanol and heat stress (Volker *et al.*, 1994, Volker *et al.*, 1992, Hecker *et al.*, 1988, Antelmann *et al.*, 1997). The induction of these genes seemed to be independent of any particular stress stimuli; thus, they were initially termed “general stress proteins”.

The subsequent link between general stress proteins and $\sigma^B$ was demonstrated by *ctc-lacZ* and *sigB-lacZ* reporter assays, showing induction of $\sigma^B$ activity by salt, heat and ethanol stress during exponential growth (Boylan *et al.*, 1993a), and by stressful conditions of the stationary phase (Boylan *et al.*, 1993b). A parallel study used pulse labelling and a 2D gel electrophoresis technique to compare the patterns of protein inductions after the imposition of heat shock (48°C for 10 min), salt stress (4% NaCl for 10 min), glucose limitation and the stationary phase (Volker *et al.*, 1994). The analysis revealed that Ctc, RsbW, GsiB and other general stress proteins with unknown functions are induced by all the stimuli. Control experiments of protein patterns in *sigB* mutants after heat shock confirmed that the induction of the stress-related genes was $\sigma^B$-dependent, linking $\sigma^B$ with the general stress response (Holtmann *et al.*, 2004, Volker *et al.*, 1994).

To define members of $\sigma^B$ regulon expression, profiles under a range of stressful conditions that were assumed to induce $\sigma^B$ activity were investigated. Global approaches were utilised to find proteins that are no longer starvation- or stress-
inducible in sigB mutants. Almost 50 σB-dependent genes were identified by proteomics after treatment with a range of stresses (heat, salt, acid, oxidative stress, puromycin; starvation for glucose, phosphate, oxygen and amino acids) (Bernhardt et al., 1999). The target promoter sequence of σB was identified for an additional 30 genes via a bioinformatic search for a specific DNA consensus sequence, after the genome of B. subtilis was sequenced (Petersohn et al., 1999). Most of the genes described as σB-dependent in the 2D gel analysis and promoter screening were also found among 125 identified by subsequent gene array-based analyses (Petersohn et al., 2001). The introduction of global transcriptional profiling created a comprehensive picture of the regulatory role of σB in adaptation to stress (ethanol, salt, acid, heat, cold, H₂O₂), and provided new perspectives for the study of gene functions and σB transduction pathways (Wilks et al., 2009, Price et al., 2001, Helmann et al., 2003, Brigulla et al., 2003, Budde et al., 2006).

To verify the dynamics of changes in protein patterns, and to observe the timing of σB-dependent expression, the levels of mRNA in the stress genes (ctc, rsbW, sigB, rsbX and gsiB) were quantified after the imposition of stresses (heat, salt and oxygen limitation) by slot-blot hybridisation with digoxigenin-labelled probes (Volker et al., 1994). A similar pattern of increase in transcript levels was observed for all σB-dependent genes with maximal induction of σB activity within no more than 15 min after stress was applied. Rapid induction of σB-dependent stress response was also reported by measuring β-galactosidase activity from a single-copy transcriptional fusion of sigB-lacZ after the addition of 0.3M NaCl and 4% ethanol (Boylan et al., 1993a). Interestingly, a transcriptomic approach looking at global changes in mRNA levels on a time-resolved scale after an osmotic upshift (0.4 M NaCl) reported that the change in the expression pattern was not only rapid, but also transient when compared to the continuous propagation of the cells in the presence of osmotic stress at the optimal temperature of growth (Steil et al., 2003).

To observe the influence of low temperatures on σB activity, β-galactosidase production was monitored using a ctc-lacZ reporter over slow growth from exponential to stationary phase (up to 240 hours), at 16°C (Brigulla et al., 2003). Induction of σB activity after a temperature downshift from 37°C to 16°C was not observed immediately after stress imposition, but peaked in the mid-exponential
phase when cells doubled twice. Interestingly, a similar pattern was observed in mutants of regulatory proteins RsbU, RsbP and RsbV, suggesting that, at low temperatures, $\sigma^B$ might be activated via an alternative branch of signal transduction. RsbV-independent activation of $\sigma^B$ was confirmed with gsiB-gfp fusion and visualised with fluorescent microscopy, but the additional regulatory pathway remains to be elucidated (Holtmann et al., 2004).

Most recently, the popularisation of fluorescent proteins opened a new area of research and allowed the visualisation of gene expression at a single cell level in a genetically identical bacterial population. To monitor sporulation and general stress response ongoing in a population of B. subtilis cells starving for glucose, fluorescent reporters ($\text{amyE}::\text{P}_{\text{spoIIA}}-\text{cfp}$ $\text{P}_{\text{ctc}}-\text{yfp}$) were developed (Hornstra et al., 2009). Within one cell, $\sigma^B$-dependent response (YFP production) was not induced if the cell was undergoing early sporulation (CFP production), suggesting that both responses are not induced at the same time and are mutually exclusive. The study demonstrated heterogeneity of response to nutrient starvation and suggested the possibility of a bistable switch between the two cellular processes, a phenomenon previously reported in B. subtilis and other bacteria (Dubnau and Losick, 2006). Moreover, the time-lapse microscopy approach with $\text{P}_{\text{sigB}}$-YFP reporter revealed that the $\sigma^B$-dependent response is stochastic and unsynchronised across the population, and does not depend on the level of induction observed in mother bacterial cells (Locke et al., 2011). Thus, in B. subtilis, a noise-driven distribution of different states is observed, and this might contribute to adaptability to the changing environment.

The role of the stochasticity of cellular processes in the regulation of $\sigma^B$ was further investigated in B. subtilis under energy stress (Locke et al., 2011). The study reported that, in bacterial cells, $\sigma^B$ is activated by energy stress in discrete pulses, and that pulse frequency is increasingly proportional to the level of stress. The frequency of stress response activation is modulated by a combination of noise in the activity of a kinase–phosphatase pair (RsbW-RsbP) and the likelihood of crossing the threshold of a sensitive phosphorylation switch. Interestingly, the correlation between concentrations of inducer and the extent of $\sigma^B$ activity was previously described for the environmental pathway in the structural study of the stressosome (Marles-Wright and Lewis, 2008). A proportional induction of $\sigma^B$ activity in the presence of
increasing concentrations of ethanol or NaCl was reported by ctc-lacZ fusion, and was attributed to multiple copies of RsbT present in the sensory molecules distributed throughout the cell. Sensitive phosphorylation of the core, consisting of multiple copies of RsbR- RsbR_p-RsbS by RsbT, causes RsbT release and activation of downstream signaling (Eymann et al., 2011). Thus, an additional noise-dependent phosphorylation switch might also be present in the environmental pathway, but this speculation requires further study.

In conclusion, the range of methodological approaches used allowed the conditions and timing characteristics required for activation of σB in B. subtilis to be uncovered. Population-based studies average σB-dependent responses at the RNA and protein levels from cells grown together under the same conditions. These techniques dominated above single cell analysis in B. subtilis. New technical approaches becoming more available might help to elucidate missing information regarding mechanisms of heterogeneity of σB activity, initial stress sensing or alternative activation cascades. Despite some unanswered questions, extensive studies carried out to understand σB induction in B. subtilis can serve as a model for analysis of many still poorly understood regulatory factors and σB in other bacteria.
1.5. SIGMA FACTOR $\sigma^B$ IN L. MONOCYTOGENES

1.5.1. The role of $\sigma^B$ in stress response in L. monocytogenes

$\sigma^B$ is one of four alternative sigma factors described in L. monocytogenes (section 1.3.2). It is involved in the regulation of the expression of genes involved in stress response and virulence (O'Byrne and Karatzas, 2008). $\sigma^B$ was first described in L. monocytogenes by two independent research groups in 1998 (Becker et al., 1998, Wiedmann et al., 1998). Stress-sensitive phenotypes for mutants lacking $\sigma^B$ have been reported for a range of conditions, including osmotic pressure (Becker et al., 1998), low pH (Wiedmann et al., 1998), high pH (Giotis et al., 2010, Giotis et al., 2008), cold shock and heat shock (Bayles et al., 2000, Becker et al., 1998), high hydrostatic pressure (Wemekamp-Kamphuis et al., 2004c), blue and red light (Ondrusch and Kreft, 2011), low O$_2$ (Toledo-Arana et al., 2009), bile (Begley et al., 2005, Zhang et al., 2011), antibiotics (Begley et al., 2006, Zhou et al., 2012, Shin et al., 2010b, Palmer et al., 2009), hydrogen peroxide (Becker et al., 1998), ethanol (Ferreira et al., 2001), disinfectants (van der Veen and Abe, 2010), detergents (Ryan et al., 2008a) and starvation (Herbert and Foster, 2001, Ferreira et al., 2001). The involvement of $\sigma^B$ in tolerance to freeze-thawing cycles (Wemekamp-Kamphuis et al., 2004c) and biofilm formation under continuous-flow or static conditions were also reported (van der Veen and Abe, 2010). Many of these environments are encountered by the pathogen in nature, during food processing and at various stages of infection, indicating the role of $\sigma^B$ in survival and pathogenesis of L. monocytogenes.

To understand the phenotypes reported for sigB mutant, ongoing research aimed at defining the full extent of the $\sigma^B$ regulon. Three years after the discovery of $\sigma^B$, the genome of L. monocytogenes EGD-e was published (Glaser et al., 2001). Since then, a number of groups used transcriptomics and proteomics to describe more than 200 $\sigma^B$-regulated genes that were differentially expressed in L. monocytogenes wild-type and the corresponding $\Delta$sigB mutant strain grown under a range of stresses, including osmotic stresses, extreme temperatures, low pH or infection-related environments (Abram et al., 2008b, Kazmierczak et al., 2003, van der Veen et al., 2007, Toledo-Arana et al., 2009). Comprehensive elucidation of a full set of $\sigma^B$-dependent genes,
together with studies addressing the role of selected genes, helped develop understanding of its involvement in stress tolerance, metabolism and virulence.

1.5.1.1. Osmotic stress response, and the role of σB

*L. monocytogenes* is able to grow in environments of high osmolarity (e.g. at NaCl concentrations of up to 2 M), and can survive for extended periods of time in media containing 3 M NaCl (Cole *et al*., 1990). Fundamental to this trait is a mechanism allowing modulation of optimal turgor pressure via the uptake of specific compatible solutes. *L. monocytogenes* is able to accumulate the compatible solutes glycine-betaine (betaine) and carnitine, through transport systems (Csonka, 1989). Under hyperosmotic conditions, a loss of water from the cell, and an increase in the concentrations of intercellular metabolites, are observed. The solutes can counteract the high extracellular solute concentration and maintain optimal water activity to ensure cellular integrity, essential physiological processes and nutrient uptake for the support of bacterial growth.

The presence of an ATP-dependent carnitine transport system, and an ATP-dependent salt-inducible betaine transporter, together with a sodium ion-driven betaine transport system, were first suggested in *L. monocytogenes* Scott A strain by biochemical studies (Gerhardt *et al*., 1996, Verheul *et al*., 1995). Physiological observations were evaluated at the genetic level via the cloning of the genes of three distinct transporters, BetL, Gbu, and OpuC, that have been identified as achieving compatible solute accumulation in *L. monocytogenes*. The uptake of betaine occurs through the ATP-dependent Gbu transporter and the sodium-dependent BetL transporter (Patchett *et al*., 1992, Sleator *et al*., 1999), encoded by the *gbuABC* and *betL* operons, respectively. OpuC (encoded by *opuCA, opuCB, opuCC* and *opuCD*) is responsible for the accumulation of carnitine (Angelidis *et al*., 2002, Fraser *et al*., 2000). Multiple mutations in *betL, gbuA*, and *opuC* were created, and confirmed that OpuC (Fig. 1.8) is the major carnitine transporter in *L. monocytogenes* (Angelidis and Smith, 2003).
Fig. 1.8. Schematic organisation of the OpuC system in *L. monocytogenes* EGD-e. (A) The *opuC* operon, preceded by $\sigma^B$-dependent promoter(s), consists of four ORFs, shown as arrows, together with their predicted sizes and function. (B) The model for the OpuC carnitine transporter, consisting of four proteins encoded by the *opuC* operon. OpuCA (a) is a hydrophilic protein, and encodes an ATPase subunit of the transporter. OpuCB; (b) is a hydrophobic, membrane-spanning protein, a permease subunit, OpuCC; (c) is a hydrophilic protein, and a substrate-binding protein, tethered to the cytoplasmic membrane. OpuCD is a hydrophobic polypeptide believed to act as the second permease subunit, in conjunction with OpuCB.

Based on homology to the carnitine transport system described in *B. subtilis*, a second potential carnitine transporter OpuB (encoded by *lmo1421* and *lmo1422*) was suggested to exist in *L. monocytogenes* (Fraser et al., 2000). These genes were later described as the bile exclusion system BilE, and were shown not to be involved in carnitine transport (Sleator et al., 2005). However, in the absence of a functional OpuC system, significant carnitine transport was still observed, suggesting the existence of an alternative transport pathway in *L. monocytogenes* (Fraser et al., 2000). Additional compatible solutes used by *L. monocytogenes* were also reported, and include proline-betaine, acetyl-carnitine, $\gamma$-butyrobetaine, and 3-dimethylsulphoniopropionate (Bayles and Wilkinson, 2000). Proline, which has been shown to act as a major compatible solute of bacteria, does not play a significant role in *L. monocytogenes* osmotolerance (Sleator et al., 2003).
In the first study that demonstrated the involvement of σB in the stress response of *L. monocytogenes*, the magnitude of the response was greatest after the addition of salt (Becker *et al*., 1998). The involvement of σB in the response to osmotic shock in *L. monocytogenes* was demonstrated soon after, via increased σB activity and decreased growth of the mutant lacking functional *sigB* (Sleator *et al*., 1999). The same study reported impaired accumulation of betaine in *sigB* mutant. Indeed, *betL* is preceded by a putative σB promoter, but its transcription occurs mostly in a σA-dependent manner, since it was shown not to be dependent on σB (Fraser *et al*., 2003). Additionally, one of two promoters upstream from *gbuA*, designated *gbuAP2*, was reported to be σB-dependent (Cetin *et al*., 2004). Thus, impaired uptake of betaine in the absence of σB might be due to decreased Gbu activity, rather than BetL, but further clarification of the role of σB in the regulation of betaine transporters will be necessary. The putative σB promoter was suggested upstream of *opuCA* (Fraser *et al*., 2000) and σB-dependence of the OpuC system was demonstrated in subsequent study (Fraser *et al*., 2003). The σB promoter was later identified 58bp upstream from the OpuC operon, by RACE-PCR (Kazmierczak, 2003). An additional σB-dependent promoter was mapped to position 43bp further upstream, by detecting both a primer extension product absent in Δ*sigB* and alignments of the sequences of known σB-dependent promoters (Cetin *et al*., 2004).

An induction of a transcription of σB-dependent genes was observed via a partial gene array study, which investigated expression of 208 genes of *L. monocytogenes* 10403S exposed to osmotic stress (0.5 M KCl), or grown to stationary phase (Kazmierczak *et al*., 2003). The study predicted a σB-dependent promoter, with a hidden Markov model (HMM) upstream of 166 genes, and identified 55 genes with 1.5-fold higher expression in the wild-type than the *sigB* mutant. A whole-genome array analysis comparing σB regulon of *L. monocytogenes* 10403S and *L. innocua* grown in the presence of 0.3 M NaCl reported 168 genes as positively regulated by σB in *L. monocytogenes*, and only 65 in non-pathogenic species (Raengpradub *et al*., 2008). More recent analysis, which also used a whole-genome array, showed more than 200 genes regulated by σB, and revealed that the σB regulon included 7.6% of genes in the *L. monocytogenes* EGD-e genome (Hain *et al*., 2008). The role of the σB regulon in the presence of 0.5 M NaCl was evaluated in a proteomic study, using a combination of 2DE and iTRAQ (Abram *et al*., 2008a). A total of 38 genes were
reported to be $\sigma^B$-dependent, with some (OpuC, HtrA) previously shown to be involved in osmotolerance.

The involvement of the selected osmotic transporter systems, which are regulated at least partly by $\sigma^B$, in response to cold shock and growth in the presence of low temperatures, was also reported (Chan et al., 2007a). An overlap between osmotolerance and cryotolerance observed in L. monocytogenes was attributed to the ability to accumulate protective compatible solutes by three transport systems, described above. However, some studies suggested that the regulation of the expression of transporter genes might differ between low and optimal temperatures, and that even the well-known $\sigma^B$-dependent genes might be transcribed in a $\sigma^B$-independent manner (Chan et al., 2007a). The role of $\sigma^B$ at a low temperature is discussed further in Chapter 4.

1.5.1.2. The role of $\sigma^B$ in response to other stresses

Acid

L. monocytogenes is able to adapt to a low pH environment in acidified foods, upon passage through the GI tract and in the host cell phagosome. An acid tolerance response (ATR) in L. monocytogenes is mostly determined by the glutamate decarboxylase (GAD) system, the arginine deiminase (ADI) system, the F0F1-ATPase and general stress proteins such as DnaK, GroEL, Htra and the Clp ATPases (Ryan et al., 2008b). $\sigma^B$ contributes to acid resistance primarily through the GAD system, which exchanges extracellular glutamate for an intracellular $\gamma$-aminobutyrate (GABA$_\gamma$) (Cotter et al., 2005b). Inside the cell, the glutamate is decarboxylated to GABA$_\gamma$, a proton is consumed, preventing acidification of the cytoplasm. Most of strains of L. monocytogenes encode three distinct glutamate decarboxylases ($gadD1$, $gadD2$ and $gadD3$), and two glutamate-GABA antiporters ($gadT1$ and $gadT2$), organised in three separate loci: $gadD1T1$ ($lmo0447-8$), $gadT2D2$ ($lmo2362-3$) and $gadD3$ ($lmo2434$).

Putative $\sigma^B$ promoters were identified upstream of $gadT2D2$ and $gadD3$, and RT-PCR analysis confirmed that their transcription is $\sigma^B$-dependent in L. monocytogenes.
EGD-e (Wemekamp-Kamphuis et al., 2004c). The transcription of gadT2D2 and gadD3, induced by acid, is abolished in ΔsigB mutant. gadT1 expression is reduced, and gadD1 is increased in the absence of σB, suggesting an alternative mechanism of regulation compensating for the absence of others controlled by σB. Subsequently, gadT2D2 has been shown to be induced during very low pH exposure (pH 2.8), and in stationary phase, while gad1 is activated in exponential phase, and at mildly acidic pH levels (pH 5.1) (Cotter et al., 2005b). The survival of ΔsigB mutant exposed to either pH-adjusted BHI (pH 2.5) or synthetic gastric fluid (pH 2.5) was lower than the survival of wild-type cells at every point of growth, from exponential to stationary phase. However, the survival of both ΔsigB and wild-type L. monocytogenes strains was induced by a similar order of magnitude upon entry into stationary phase, suggesting that the growth phase-dependent acid resistance mechanism is regulated in a σB-independent manner (Ferreira et al., 2003).

Antimicrobials – disinfectants and antibiotics

The role of σB in response to antimicrobial treatment was mostly attributed to its ability to modulate membrane characteristics and induce cell surface alterations to ensure cell integrity. Levels of the sigB gene were shown to be increased in the presence of sublethal concentrations of quaternary ammonium compounds (QAC), including benzalkonium chloride (BC), cetylpyridinium chloride (CPC) and sodium dodecyl sulfate (SDS), which are common components of industrial cleaning agents (Ryan et al., 2008a). σB has been shown to be essential for survival in the presence of lethal doses of BC, CPC and SDS. Similarly, survival of the biofilm forming cells of L. monocytogenes under the BC and peracetic acid treatments was partly attributed to σB (van der Veen and Abee, 2010).

The contribution of σB in response to treatment by cell envelope-acting antibiotics and bacteriocins (ampicillin, penicillin, nisin and lacticin) was reported in L. monocytogenes (Begley et al., 2006). A link between nisin resistance and the acid stress response of L. monocytogenes was suggested in the study investigating the role of the GAD system in antimicrobial response (Begley et al., 2010). The study showed that gadD1 may contribute to intracellular ATP pools counteracting the outflow caused by the formation of pores within the membrane, or might be related to the controlling of the cell’s proton motive force. The role of the σB-dependent
gene lmo2570, which is highly similar to the bacteriocin immunity gene sdpI, from B. subtilis, in resistance to bacteriocins, was ruled out in L. monocytogenes (Palmer et al., 2009). However, tolerance to nisin and SdpC were shown to be both $\sigma^B$- and $\sigma^L$-dependent. In the most recent proteomic study, 18 vancomycin-inducible $\sigma^B$-dependent proteins were described (Shin et al., 2010b). The proteins identified were mostly cell wall–associated, including transporter proteins. Interestingly, 8 of these have not been previously reported as members of the $\sigma^B$ regulon in previous studies, suggesting a specific stress-related $\sigma^B$-dependence.

Blue and red light

Light is a ubiquitous environmental factor, and the ability to sense and respond to this stimulus might be useful in preparing a bacterial cell for desiccation, high temperatures or radiation exposure, causing photochemical damage to cellular components. LOV, the only characterised stress sensory domain that was described thus far in L. monocytogenes, was shown to perceive blue light, and to activate $\sigma^B$ upon illumination. The domain is found within one of the RsbR paralogues – Lmo0799, the presumptive homologue of YtvA (Ondrusch and Kreft, 2011). The LOV domain was named after the type of signal detected (light, oxygen and voltage), and it is a subclass of PAS domains (name derived from three transcriptional regulators: Per, Arnt and Sim, which contain such a domain). In Lmo0799, LOV is the N-terminal input domain, and it is linked to the C-terminal sulfate transporter and an anti-sigma factor antagonist (STAS) domain. The photoactive LOV domain non-covalently binds flavin mononucleotide (FMN). A signal transduction process starts with a covalent interaction of a single molecule of FMN to a conserved cysteine in the LOV domain, upon illumination (Losi, 2004).

It was demonstrated that L. monocytogenes expression of selected $\sigma^B$-dependent genes was induced by blue light, and that induction was abolished in the sigB background (Ondrusch and Kreft, 2011). Some of the genes tested, which are involved in virulence (inlA, inlB and prfA), were shown to be activated by light in a $\sigma^B$-dependent manner. Furthermore, the invasiveness of L. monocytogenes into Caco-2 enterocytes was 2-fold higher if bacteria were exposed to blue light for 2 hours. Interestingly, a combination of blue light exposure with 0.3 M NaCl stress increased the expression of inlA and inlB 5-fold and 6.5-fold, respectively. Perception of red
light was also reported to activate $\sigma^B$ in *L. monocytogenes*. However, the red light effect on $\sigma^B$ was lower than the blue light induction, but was surprisingly higher in the *lmo0799* background than in wild-type. The mechanism of $\sigma^B$ induction by a higher energy light is assumed to differ between *B. subtilis*, where it is sensed via the RsbPQ pathway, and *L. monocytogenes*, where this pathway is missing (Avila-Perez *et al.*, 2010).

**Bile**

Bile is a biological detergent synthesised in the liver and secreted into the digestive tract for the emulsification and solubilisation of lipids. Thus, it has the potential for antimicrobial activity, and can lyse the bacterial cells ingested. *L. monocytogenes* is able to colonise a gall bladder and tolerate high concentrations of bile (Hardy *et al.*, 2004). The bile resistance in *L. monocytogenes* is attributed to two mechanisms. The first is based on the enzymatic modification of a chemical form of bile salts by bile salt hydrolase, encoded by *bsh* (*lmo0446*) (Dussurget *et al.*, 2002). Hydrolysis of the amide bond liberates the glycine or taurine from the steroid core, creating the form which is less toxic to the bacterial cells. The second mechanism involves an active exclusion of bile molecules by transporters, mainly the bile exclusion system, BilE (Begley *et al.*, 2002, Sleator *et al.*, 2005). The role of the efficient transport of compatible solutes OpuC, BetL, and Gbu, previously described as being involved in the osmotolerance and cryotolerance of *L. monocytogenes* in response to bile, has been shown recently (Watson *et al.*, 2009).

$\sigma^B$ plays a central role of in the regulation of gene expression in response to bile stress. The $\Delta sigB$ strain is sensitive to bile salts, and the survival of the mutant exposed to high concentrations of bile was reported to be strongly decreased (Begley *et al.*, 2002, Zhang *et al.*, 2011). The involvement of $\sigma^B$ in the regulation of bile-associated loci is also experimentally proven. *bsh* possesses a $\sigma^B$ promoter upstream of the gene, and its expression in the $\Delta sigB$ strain is decreased (Kazmierczak *et al.*, 2003, Sue *et al.*, 2003). BilE also contains a $\sigma^B$-specific promoter-binding site 84 bp upstream of the *bilE* (*lmo1421* and *lmo1422*) operon (Sleator *et al.*, 2005), and is regulated in the $\sigma^B$-dependent manner (Fraser *et al.*, 2003). The role of $\sigma^B$ in the regulation of the main osmolyte uptake systems OpuC, BetL and Gbu, was discussed previously (Section 1.5.1.1). A disruption of the selected $\sigma^B$-regulated loci (*bsh*, *pva*, *lmo0446*).
opuC) causes decreased survival in the intestine, and reduced colonisation (Begley et al., 2005, Watson et al., 2009). Moreover, σB-dependent upregulation of these genes is observed in vivo in the intestine (Toledo-Arana et al., 2009).

1.5.1.3. The role of σB in virulence

Based on the previously described transcriptomic and proteomic study, it is clear that the σB regulon includes genes that are directly involved in the pathogenesis of L. monocytogenes (Milohanic et al., 2003, Kazmierczak et al., 2003, Raengpradub et al., 2008). Indeed, the master regulator of gene expression, PrfA, is itself transcribed, at least partly, in the σB-dependent manner (Nadon et al., 2002) (Section 1.3.1). A subcategory of 53 PrfA-regulated genes was reported to possess the σB consensus promoter (Milohanic et al., 2003), and later, 45 of these genes were shown to be expressed in a σB-dependent manner (Hain et al., 2008). Thus, it is not unexpected that the ΔsigB mutant strain, as well as several mutants lacking σB-dependent genes (inlAB operon), display reduced invasiveness of epithelial cell lines (Kim et al., 2004).

In vivo experiments demonstrated that the ΔsigB strain has a much lower ability to invade guinea pigs when introduced via the oral route, but not when introduced intravenously (Garner et al., 2006b). Thus, the role of σB in the invasiveness of L. monocytogenes was also related to survival, due to the activation of the stress response against gut-specific signals, including low pH levels (stomach), high osmolarity (low intestine) and the presence of bile salts (gall bladder, low intestine). The main carnitine transporter, OpuC, has been also shown to be required for full virulence when L. monocytogenes was administered orally (Sleator et al., 2001, Wemekamp-Kamphuis et al., 2002). Conditions simulating the GI tract influenced survival of the ΔsigB strain (Ferreira et al., 2001, Ferreira et al., 2003), and upregulated the expression of the σB regulon in wild-type (Sue et al., 2004). Transcription of sigB was also increased in the intestine during murine infection (Begley et al., 2005)

A comprehensive genome-wide pattern of transcription in L. monocytogenes exposed to the GI tract-related environments, compared to exponentially grown bacteria at optimal temperatures, was examined by tiling arrays (Toledo-Arana et al., 2009). In
this approach, 25-bp nucleotide fragments, with 9-bp overlaps covering both strands of entire genome of *L. monocytogenes* EGD-e, were used. RNA was extracted from the wild-type strain, ΔsigB, ΔprfA and Δhfq strains, which were exposed to 30°C, low oxygen, or grown to stationary phase. Additionally, RNA from the bacteria isolated from the intestine of mice 24 hours post–inoculation, and after 30 and 60 min of incubation within blood, was also isolated for further expression profiling and comparisons. σB was found to modulate expression of 232 genes within the intestine, in contrast to PrfA, which was shown to play a minor role at early stages of infection. Interestingly, the switch between the two transcriptional regulators in the gene expression was observed within blood. Thus, σB and PrfA were shown to play distinct regulatory roles during listeriosis, with the first dominating in the GI tract, and the second at later phase of infection. Furthermore, deletion of σB influenced the expression of selected sRNA to a higher extent than the loss of either PrfA or Hfq, but closer examination of their overlapping regulatory roles during the infectious lifecycle of *L. monocytogenes* deserves further attention.

### 1.5.1.4. The role of σB in metabolism

Several transcriptomic studies of the σB regulon, mentioned above, aimed to identify genes transcribed in the σB-dependent manner, and group them into functional categories. The role of σB in metabolism is hard to distinguish from its role under stress (e.g. starvation), as the pathways are presumably not exclusive components of the complex response. However, among σB-dependent genes identified by microarrays in *L. monocytogenes* 10403S cells grown to stationary phase, or under salt stress, the group of metabolic-related genes was the second most abundant category (Raengpradub *et al*., 2008). Only genes with unknown functions were more highly represented in the analysis. The group of genes involved in carbohydrate transport and metabolism was also highly represented in the transcriptomic study of *L. monocytogenes* EGD-e investigated over growth in complex BHI media (Hain *et al*., 2008). Genes encoding putative sugar uptake systems (*lmo0196, lmo0781-84*), enzymes involved in glycolytic pathways (*lmo2695-97, lmo2205*), or enzymes taking part in cell wall turnover (*lmo0956-7*), are only selected examples of metabolic genes that were reported to be part of the σB regulon.
One of the proteomic studies also reported that 22 out of 38 $\sigma^B$-regulated genes
identified in the analysis can be categorised into the group of genes involved in
metabolism (Abram et al., 2008b). Further investigation revealed that the $\Delta\sigma^B$
strain possesses a decreased ability to utilise alternative carbon sources. Three
proteins involved in glycerol metabolism were also reported to be downregulated in
the $\Delta\sigma^B$ background, including Lmo2695, Lmo2696 and Lmo2697. Interestingly,
13 out of 22 genes involved in metabolism were reported to be negatively regulated
by $\sigma^B$, with higher spot intensity observed in the $\Delta\sigma^B$ strain. Similarly, in the
microarray study lower transcript levels were observed in wild-type than in the $\Delta\sigma^B$
strain, mostly in stationary phase, for more than 100 genes (Hain et al., 2008). This
might be partly explained by the need to maintain energy costs and silence some of
the metabolic pathways when cells are not growing, after they enter stationary phase.
Moreover, the classification of genes into functional categories is mostly made on
homology, and the functions of the majority of the genes require further structural
and biochemical studies.

1.5.2. The present model of regulation of $\sigma^B$ in *L. monocytogenes*

1.5.2.1. Transcriptional regulation of $\sigma^B$ expression

The current mechanism of $\sigma^B$ regulation in *L. monocytogenes* is based largely on the
model extensively studied in *B. subtilis* (Hecker et al., 2007). Comparative genomics
showed that the operon containing $\sigma^B$ and its regulatory proteins (Fig. 1.9) is
conserved between *L. monocytogenes* and *B. subtilis*, suggesting similar functional
roles for the corresponding genes (Ferreira et al., 2004). Thus, $\sigma^B$ is encoded by the
$\sigma^B$ gene, which is the seventh open reading frame in an eight-gene operon ($rsbR-
rsbS-rsbT-rsbU-rsbV-rsbW-sigB-rsbX$). The operon additionally contains seven
regulation of $\sigma^B$ (Rsb) genes whose products are thought to be directly involved
in the activation of $\sigma^B$ at post-translational level, through complex protein-protein
interactions.
Fig. 1.9. Organisation of the *sigB* operon in *L. monocytogenes*. The identities of the amino acid sequence between homologues from *B. subtilis* are shown as the percentages. Each gene is scaled based on Listilist. Transcription of the genes occurs from the promoters, indicated by ‘P’ (Toledo-Arana *et al*., 2009, Becker *et al*., 1998, Ferreira *et al*., 2004).

The downstream part of the operon (*rsbV-rsbW-sigB-rsbX*) is transcribed in a $\sigma^B$-dependent manner from the promoter (P$_2$), located upstream of *rsbV*. Thus, increased activity of $\sigma^B$ causes the transcription of the *sigB* gene, creating a positive feedback loop in response to conditions stimulating $\sigma^B$. Transcription from the P$_2$ can be induced by a range of stresses, including the stationary phase, NaCl, ethanol and heat shock (Becker *et al*., 1998). All eight genes are assumed to be co-transcribed from the promoter (P$_1$), which in *B. subtilis* is dependent on housekeeping sigma factor $\sigma^A$, identified upstream of *rsbR* (Wise and Price, 1995). However, no experimental data proves the existence of this transcript in *L. monocytogenes*. A recent comprehensive transcriptomic study suggested that the eight-gene *sigB* operon is co-transcribed from another promoter (P$_0$) with the two upstream genes *lmo0887* and *lmo0888* (Toledo-Arana *et al*., 2009). In *Staphylococcus aureus*, a module located immediately upstream of the *sigB* operon is transcribed with the *sigB* operon, and encodes a toxin-antitoxin system, MazEF, involved in the regulation of $\sigma^B$ (Donegan and Cheung, 2009). However, no further data explains the significance of the co-transcribed genes to the regulation of $\sigma^B$ in *L. monocytogenes*. 
1.5.2.2. Post-translational regulation of $\sigma^B$ activity

The availability of $\sigma^B$ to participate in transcription is assumed to be orchestrated in *L. monocytogenes* through the complex protein-protein interactions and the phosphorylation status of the regulatory proteins, as it is in *B. subtilis* (Section 1.4.1.). In summary, in the absence of activation stimuli, $\sigma^B$ is sequestered by its anti-sigma factor RsbW, and is not able to interact with the RNAP. When a stress stimulus occurs, dephosphorylation of anti-anti-sigma factor RsbV is triggered. The dephosphorylated RsbV binds to RsbW, releasing $\sigma^B$ and providing an opportunity for it to interact with the RNAP. Thus, the phosphorylation state of RsbV determines the activity of $\sigma^B$. To date, it has not been evaluated whether the same members of the $\sigma^B$ activation pathway described in *B. subtilis* are involved in analogous signal transmission cascade in *L. monocytogenes*.

In *B. subtilis*, the phosphorylation state of RsbV is tightly regulated by two distinct phosphates, RsbP and RsbU. RsbU is activated in response to environmental stress stimuli, via the RsbRSTU pathway (Kang *et al.*, 1996), while RsbP senses energy-related stress, and requires a second protein, RsbQ (Vijay *et al.*, 2000). Moreover, a third phosphatase-independent pathway was reported to be induced at low temperatures (Brigulla *et al.*, 2003). The members of the RsbRSTU regulatory cascade are encoded in the *L. monocytogenes* genome, upstream of the *sigB* operon, as in to *B. subtilis*. Four additional paralogues of RsbR (Lmo0161, Lmo0799, Lmo1642 and Lmo1842) can be found within the genome of *L. monocytogenes*. Moreover, the paralogues of RsbR are also found in the genome of non-pathogenic *L. innocua*, suggesting that their role in *L. monocytogenes* is not directly related to virulence (Pane-Farre *et al.*, 2005, Losi, 2004).

Little experimental data is available to support the role of stressosome elements in the activation of $\sigma^B$. One genetic study suggested that energy and environmental stresses are sensed via the RsbRSTU pathway in *L. monocytogenes*. Both $\Delta rsbT$ and $\Delta rsbV$ demonstrated survival reductions similar to that of the $\Delta sigB$ strain, under environmental stress and energy stress (Chaturongakul and Boor, 2004). To reflect $\sigma^B$ activity, the transcript levels of known $\sigma^B$-dependent genes were measured in response to both types of stress in $\Delta rsbT$ and $\Delta rsbV$, and were similar to levels in the
ΔσB strain (Chaturongakul and Boor, 2006). The role of RsbU phosphatase activity and the kinase activity of RsbT were evaluated in *L. monocytogenes* by an in-frame deletion mutation in *rsbU* and a missense (D80N) mutation in *rsbT* (Shin et al., 2010a). These mutations did not abolish the ability of protein-protein interactions required for proper stessosome assembly (Marles-Wright and Lewis, 2008). RsbT and RsbU were both required for the induction of σB in response to a wide range of stresses, including salt, acid, ethanol, wall-acting antibiotics, and temperature extremes (Shin et al., 2010a). The role of RsbT under energy stress is less clear, as σB activity is induced in *rsbT* mutant while it is abolished in the *rsbU* background.

The role of stress sensing by RsbR paralouges in *L. monocytogenes* was evaluated for Lmo0799. In the study investigating stress response to blue and red light, Lmo0799 was reported to be a functional homologue of the photoreceptor YtvA, from *B. subtilis* (Ondrusch and Kreft, 2011). In *L. monocytogenes*, Lmo0799 is involved in the activation of the transcription of σB-dependent genes by blue light, due to the interactions within the LOV sensory domain, described before in *B. subtilis* (Gaidenko et al., 2006) (Section 1.5.1.2). However, Lmo0799-independent light induction of σB is also observed, suggesting that an alternative receptor for light-dependent activation of σB exists or that light causes secondary stresses which are then sensed via another sensor (Ondrusch and Kreft, 2011).

Interestingly, the expression of *lmo0799* is co-regulated by a riboswitch, an RNA region that is specific to lysine (Toledo-Arana et al., 2009). The LysRS riboswitch is located 280 nucleotides upstream of a lysine transporter gene (*lmo0798*), and immediately downstream from the *lmo0799* gene. Additionally, LysRS can function as a 3’UTR regulator, and can regulate expression of the upstream gene *lmo0799*. Lysine depletion leads to the formation of the anti-terminator structure within the LysRS, and results in the LysRS-*lmo0798* transcript. *lmo0799* lacks an intrinsic 3’ terminator, and a long *lmo0799*-LysRS-*lmo0798* transcript is generated in the absence of lysine. In the presence of lysine, a stable tertiary structure is formed, and the expression of the transporter gene is blocked, while transcripts for Lmo0799-LysRS are detected. Thus, *lmo0799* is transcribed in a lysine-independent manner.
In contrast to the RsbRSTU regulatory cascade, no closely related genes have been identified in the *L. monocytogenes* genome for the RsbQR pathway (Ferreira *et al*., 2004). The presence of a putative RsbQ homologue, designated *orfX* (*yugF*), was reported, but there is no experimental data suggestive of OrfX’s involvement in the regulation of $\sigma^B$ (Brondsted, 2003). Since no homologue of RsbP is encoded in the genome of *L. monocytogenes*, both environmental and energy stresses are proposed to be sensed through RsbU (Shin *et al*., 2010a). Furthermore, when *rsbR* from *L. monocytogenes* was expressed in *B subtilis*, the phenotype of similar $\sigma^B$ activation was observed, under both physical and nutritional stress (Martinez *et al*., 2010). Clear differences observed in the $\sigma^B$ activation pathway (Chaturongakul and Boor, 2006) emphasise the importance of further investigation of the proposed model in *L. monocytogenes*. 
1.6. PROJECT AIMS

In the model non-pathogenic organism *B. subtilis*, the alternative sigma factor $\sigma^B$ has been shown to regulate the large stress response regulon involved in survival and adaptation. Investigation of $\sigma^B$ in *B. subtilis*, ongoing since the mid-eighties, allowed the developing of a range of methodological tools and the elucidation of a comprehensive model of $\sigma^B$ regulation. A range of stimuli activating $\sigma^B$, as well as the time-resolved modulation of $\sigma^B$-dependent response, was also reported for *B. subtilis*. On the contrary, in the closely related bacterium *L. monocytogenes*, which is similarly present in soil but is also an opportunistic pathogen, there is little experimental data evaluating the mechanism of the $\sigma^B$ activation cascade, as well as the dynamics of the response. Due to the high level of conservation of the *sigB* operon between the two organisms, the current model for *L. monocytogenes* is derived from *B. subtilis*. However, it has also been reported that the degree of conservation of the $\sigma^B$ regulatory proteins varies between species, or even within the order *Bacillales*, to which *L. monocytogenes* belongs, indicating that their functions might diverge.

Clear differences in physiology between the two organisms suggest that the modulation of their stress responses might also differ, to some extent. For example, *L. monocytogenes* is non-sporulating, and can grow at much lower temperatures. Moreover, once *L. monocytogenes* is ingested into the gastrointestinal tract, it is able to switch from a saprophytic to a pathogenic lifestyle and survive within an intracellular environment. Interestingly, 75 out of the $\sigma^B$-dependent genes from *L. monocytogenes* were found to have homologues in *B. subtilis*, but only 33 of these were recognised as $\sigma^B$-dependent in *B. subtilis* (Hain et al., 2008). Thus, the divergence of the $\sigma^B$ regulon might reflect adaptation to differing niches. Due to the fact that $\sigma^B$ has been reported to be involved in the expression of virulence-related genes, comprehensive elucidation of the role and regulation of $\sigma^B$ is essential for the understanding of both the adaptability and pathogenesis of *L. monocytogenes*. Furthermore, a better insight into mechanisms of virulence and the stress response of the bacterium might help to identify putative therapeutic targets and improve pathogen control.
This study aimed to:

- review the tools and approaches that might be useful in the investigation of unanswered questions concerning the role and activity of $\sigma^B$ during the stress response of *L. monocytogenes*
- develop mRNA- and antibody-based reporter systems for monitoring $\sigma^B$ activity in *L. monocytogenes*
- identify strongly induced $\sigma^B$-dependent genes in transcriptomic studies and evaluate expression of selected genes both at the RNA level and protein level (with qRT-PCR and Western blotting)
- elucidate the conditions that activate $\sigma^B$, together with the extent and timing of that activation under selected stresses at a population level
- use a strong $\sigma^B$-dependent promoter to develop a fluorescent reporter fusion, indicating $\sigma^B$ activity, that could be measured by range of techniques to allow single cell analysis of $\sigma^B$
- investigate the role and activity of $\sigma^B$ during adaptation to the osmotic stress, the growth phase, and during low temperature growth at 4°C at a single cell level (with the reporter approach and flow cytometry)
- evaluate the role of RsbV as an anti-anti-sigma factor in the $\sigma^B$ regulatory pathway in *L. monocytogenes* EGD-e under selected conditions
CHAPTER 2

Rapid, transient and proportional activation of $\sigma^B$ in response to osmotic stress in Listeria monocytogenes

Published by Applied and Environmental Microbiology
2.1. ABSTRACT

Although the $\sigma^B$ regulon is now well defined in *Listeria monocytogenes* little is known about the way in which $\sigma^B$ activity is controlled. In this study we have measured the expression of four genes that are known to be expressed in $\sigma^B$-dependent manner in order to get insights into the nature and kinetics of $\sigma^B$ activation during conditions of osmotic stress. The data suggest that $\sigma^B$ is activated rapidly and transiently in response to osmotic stress and further suggest that the response is proportional to the magnitude of the stress encountered, features that are likely to underpin the remarkable adaptability of the pathogen.
Listeria monocytogenes is a Gram positive bacterium that is ubiquitous in the environment and is a facultative intracellular pathogen of humans (Barbuddhe and Chakraborty, 2009). Infection arise primarily in immunocompromised individuals following the ingestion of contaminated food and are associated with high mortality rates, typically 25-30% (Lorber, 1997). The remarkable adaptability of L. monocytogenes to different physical and chemical stresses underpins its ability to survive and grow in wide range of different environments. It can grow at temperatures as low as -0.4°C (Walker et al., 1990), it can survive over a wide pH range (Vasseur et al., 1999), it is extremely tolerant to bile (Hardy et al., 2004, Watson et al., 2009), and it can grow in the presence of salt concentrations as high as 2 M (Cole et al., 1990). In recent years it has become clear that many of these traits are partly under the control of the stress-inducible sigma factor SigB (σ^B) (Wemekamp-Kamphuis et al., 2004b), (Wiedmann et al., 1998).

Several recent studies have highlighted the role of σ^B in allowing L. monocytogenes to survive in the gastrointestinal tract, which is a prerequisite for establishing successful infection in the host (Sleator et al., 2009). Mutants of L. monocytogenes lacking sigB display decreased virulence in guinea pigs infected orally but not intravenously (Garner et al., 2006a). These mutants also display reduced rates of epithelial cell invasion, a finding that is explained by the involvement of σ^B in the transcription of the inlAB operon (Kim et al., 2004, McGann et al., 2007), which encodes internalin (InlA) and InlB, the surface proteins responsible for host cell invasion. Indeed the central regulator of virulence gene expression in L. monocytogenes, PrfA, is itself transcribed in a manner that partly depends on σ^B (Nadon et al., 2002). Further compelling evidence of the role for σ^B in the early stages of a listeriosis infection comes from a transcriptomics study that found numerous genes induced in the gastrointestinal tract to be under σ^B control (Toledo-Arana et al., 2009).

Although the importance of σ^B in stress adaptation and virulence is now well established, very little is known about how the activity of σ^B is regulated in this
pathogen. However, the high degree of conservation between the sigB locus in *B. subtilis* and *L. monocytogenes* suggests that similar regulatory mechanisms pertain in both organisms (Wiedmann *et al*., 1998, Becker *et al*., 1998). Extensive studies of the σ^B^ system in *Bacillus subtilis* have produced a detailed model accounting for the regulation of σ^B^ activity. It appears that control of σ^B^ activity is achieved primarily through a series of protein-protein interactions that ultimately control the availability of σ^B^ to interact with RNA polymerase core enzyme (Hecker *et al*., 2007). Under non-stressful conditions the activity of σ^B^ is limited by its interaction with an anti-sigma factor called RsbW. σ^B^ is deployed when RsbW interacts with an anti-anti-sigma factor called RsbV, whose activity is in-turn regulated by phosphorylation in a stress-dependent manner. The stress signals are integrated into this regulatory pathway via a high molecular protein complex called a stressosome, although the way in which stress is perceived by this complex remains unclear (Marles-Wright *et al*., 2008). Since all of the genes involved in this pathway are highly conserved in *L. monocytogenes* it seems probable that σ^B^ is regulated primarily at the post translational level in this pathogen. Indeed a number of studies provide genetic evidence to support this model in *L. monocytogenes* (Shin *et al*., 2010a, Chaturongakul and Boor, 2004).

In order to develop an understanding of the kinetics and extent of σ^B^ activation in *L. monocytogenes* we have investigated the effects of osmotic stress on the expression of four genes already known to be under σ^B^ control in this pathogen; *opuCA*, *lmo2230*, *lmo2085* and the sigB gene itself. The *opuCA* gene encodes a component of the OpuC system that is involved in osmo- and cryotolerance (Fraser *et al*., 2000, Wemekamp-Kamphuis *et al*., 2004a, Chan *et al*., 2007a), and which also plays an important role in survival during the intestinal phase of infection (Sleator *et al*., 2001), probably because it contributes to bile resistance (Watson *et al*., 2009). Its transcription is under σ^B^ control and the promoter has been mapped to a position 58 bp upstream from the start codon of *opuCA* (Fraser *et al*., 2000, Kazmierczak *et al*., 2003). The *lmo2230* gene encodes a putative arsenate reductase and its σ^B^ promoter has been mapped to a position 143 bp upstream from the start codon (Kazmierczak *et al*., 2003). This gene belongs to a category of genes that is under both σ^B^ and PrfA control (Milohanic *et al*., 2003, Chatterjee *et al*., 2006). The *lmo2085* gene encodes a putative peptidoglycan bound protein that has no homologue in the non-pathogenic
species *L. innocua* (Calvo *et al*., 2005). This gene has been shown in several studies to be expressed in a highly $\sigma^B$-dependent manner (Kazmierczak *et al*., 2003, Hain *et al*., 2008, Oliver *et al*., 2009, Abram *et al*., 2008a), and recently in gene microarray experiments we have found it be the gene most affected in a $\Delta sigB$ background (Starr and O’Byrne, unpublished data). The *sigB* gene itself is positively autoregulated; mutants lacking $\sigma^B$ fail to induce the 4-gene *sigB* operon (consisting of *rsbV*, *rsbW*, *sigB* and *rsbX*) in response to stress stimuli. The $\sigma^B$-dependent promoter is located upstream from the *rsbV* gene (Becker *et al*., 1998).

First we investigated the kinetics of $\sigma^B$ activation by following $\sigma^B$-dependent transcription after the sudden imposition of an osmotic stress. To do this 0.5 M salt (NaCl) was added to cultures of *L. monocytogenes* wild-type EGD-e and mutant derivative without functional *sigB* (Begley *et al*., 2005) grown in Brain Heart Infusion (BHI) broth at 37°C up to mid-exponential phase (OD$_{600}$ of 0.6). RNA was prepared at time intervals thereafter (0, 5, 10, 15, 20, 25 min) from cultures kept at 37°C and vigorously shaken. Real time RT-PCR was then used to measure transcript levels over this time period for each of the four $\sigma^B$-dependent genes studied (*opuCA*, *lmo2085*, *lmo2230*, *sigB*; Fig. 2.1 & Table 2.S1).
Fig. 2.1. Activation of $\sigma^B$ by osmotic upshock occurs rapidly and transiently. Relative transcript levels of $\text{sigB}$ and three other $\sigma^B$-dependent genes ($\text{opuCA}$, $\text{lm02230}$, $\text{lm02085}$) were measured in exponential-phase cells in wild-type (open symbols) and $\Delta\text{sigB}$ backgrounds (closed symbols) grown in BHI broth at 37°C. RNA extracts were prepared either immediately before the addition of 0.5M NaCl (0 min) or 5 min, 10 min, 15 min, 20 min or 25 min after osmotic upshock. Real-time determination of genes transcription levels was carried out as previously described (Karatzas et al., 2010). All transcript levels were first normalised to the corresponding 16S RNA levels (internal control) reference gene (Tasara and Stephan, 2007), with an efficiency correction included for each primer pair as previously described (Pfaffl, 2001), and then expressed as a percentage of the maximal level of $\text{sigB}$ transcript detected in the experiment (thereby allowing the relative transcript levels of the 4 genes to be compared). The values presented on the graphs are the means of three independent experiments and error bars indicate the standard deviations ($n=3$). Numbers shown above the graphs indicate statistically significant differences in relative gene expression (fold-change) for the wild-type between the stressed (+ NaCl at each time point) and non-stressed (time point zero before adding NaCl) conditions ($P<0.05$ in Student’s $t$ test). Note that the plot in part B uses a different scale on the Y-axis.
Table 2.1. Primers used in this study

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward (5'–3')</th>
<th>Reverse (5'–3')</th>
<th>Product size (bp)</th>
<th>Efficiency (E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>TGGGGAGCAAACAG</td>
<td>GATTAGTAAGGTTCTTC</td>
<td>213</td>
<td>2.27</td>
</tr>
<tr>
<td>sigB</td>
<td>CTATATTGGATTGCC</td>
<td>GCTTACAACACGTTGCATCAT</td>
<td>191</td>
<td>1.98</td>
</tr>
<tr>
<td>opuCA</td>
<td>GGATGAAGCGATTA</td>
<td>AACTGCCGCTGTAATAGAG</td>
<td>206</td>
<td>2.19</td>
</tr>
<tr>
<td>lmo2230</td>
<td>CATATTCGAAGTGCC</td>
<td>ATTGGCCTGAACTAGGTGAAT</td>
<td>169</td>
<td>2.12</td>
</tr>
<tr>
<td>lmo2085</td>
<td>GTTAATGGATATAAT</td>
<td>GTGCCTGGGTGAACCAATAA</td>
<td>177</td>
<td>2.27</td>
</tr>
</tbody>
</table>

*a* (Karatzas et al., 2010); *b* designed for this study, *c* established by five decimal serial dilutions of genomic DNA and cDNA according to the equation E=10-1/slope (Pfaffl, 2001).

A rapid induction of each of the four genes was recorded following osmotic upshock, with *lmo2230* showing the largest induction after 15 min (160-fold relative to time 0). For each gene the maximum transcript level was observed at the 15 min time interval, with a steady decrease in transcript levels thereafter, suggesting a transient activation of transcription in response to osmotic upshock. In the ΔsigB mutant there was no significant increase in transcription recorded for *opuCA, lmo2085* and *lmo2230* following osmotic upshock (Fig. 2.1 A, B & C), confirming the σ^B^-dependent transcription of these genes and indicating that σ^B^ activation occurs transiently in response to osmotic upshock. A small delayed induction of *sigB* transcription was observed in the ΔsigB mutant (detected with primers overlapping the intact part of *sigB*), presumably resulting from the activation of σ^B^-independent promoter (Fig. 2.1D). Twenty five min after the addition of NaCl the transcript level for each gene had returned to a level similar to that detected when cells were subjected to osmotic stress continuously during balanced growth (See fold-change numbers Fig. 2.S1), which was still significantly higher than the level detected in the absence of NaCl (Fig. 2.1). These data suggest that σ^B^ is activated rapidly and transiently following sudden exposure to osmotic stress. Furthermore the extent of σ^B^ activation following osmotic upshock is, for a short time, much greater than that observed during balanced growth when the same concentration of salt is present.
Fig. 2.2. Osmotic activation of $\sigma^B$ is proportional to the magnitude of the stress. Relative transcript levels of three $\sigma^B$-dependent genes ($opuCA$, $lmo2230$, $lmo2085$) and $sigB$ in the wild-type (open symbols) and $\Delta sigB$ mutant (closed symbols) grown in BHI broth at 37°C to exponential phase (OD$_{600}$ of 0.6) with a range of NaCl concentrations (0 M, 0.3 M, 0.6 M or 0.9 M). RNA extracts and cDNA were prepared as previously described (25). All transcript levels were first normalised as described in Fig. 2.1 and then expressed as a percentage of the maximal level of $sigB$ transcript detected in the experiment (thereby allowing the relative transcript levels of the 4 genes to be compared). The values presented on the graphs are the means of three independent experiments and error bars indicate the standard deviations (n=3). Numbers shown above the graphs indicate statistically significant differences in relative gene expression (fold-change) between the stressed (+ 0.3, 0.6 or 0.9 M NaCl) and non-stressed (0 M NaCl) conditions ($P<0.05$ in Student’s $t$ test).

When the transcript levels of the four genes selected ($opuCA$, $lmo2085$, $lmo2230$, $sigB$) were investigated during growth in range of salt concentrations a dramatic and proportional increase in the transcript levels occurred in each case as the level of
osmotic stress was increased from 0 to 0.9 M and cultures were grown at 37°C up to mid-exponential phase (OD₆₀₀ of 0.6) for RNA extraction. (2.. 2 & Table 2.S1).

The NaCl-induced increase in the transcript levels was not observed for any of the four genes in a background lacking σᵦ. Most substantial induction of the transcription for each of the four genes was caused by 0.9 M salt. Transcription of opuCA at 0.9 M salt was increased dramatically in the wild-type (~29 times higher relative to the 0 M NaCl control), while lmo2230 and lmo2085 showed ~98 and ~56 times higher transcription at this salt concentration, respectively. No increase in transcription was detected in the Δσᵦ mutant. Although the induction of sigB transcription by osmotic stress was largely dependent on σᵦ, a significant amount of sigB transcription was observed in the mutant background (Fig. 2.2D). This suggests that a baseline of σᵦ-independent transcription of the sigB operon is maintained under all growth conditions, which would be consistent with the requirement for a rapid transcriptional response when stress is encountered. Together these results indicate that σᵦ–dependent transcriptional activity is strongly stimulated by salt in a way that is directly proportional to the extent of the stress, suggesting that σᵦ activity can be fine-tuned according to the environment encountered.

The data presented above suggest that opuC expression is a useful means of measuring σᵦ activity in L. monocytogenes. We investigated whether the proportional expression of opuCA in response to osmotic stress could also be detected at the protein level. To detect OpuCA protein levels polyclonal antibodies were raised in chickens against the purified OpuCA protein (ATPase subunit of the OpuC transporter), as described in the supplementary material (Fig. 2.S2). Cultures of the wild-type (EGD-e) and mutant derivatives Δσᵦ and ΔopuCA were grown at 37°C with vigorous shaking in BHI broth supplemented with 0, 0.3, 0.6, or 0.9 M NaCl. Crude cell extracts were prepared from each culture, as described previously (Abram et al., 2008c) during either exponential phase (OD₆₀₀ of 0.6) or stationary phase (16 h culture) and the levels of OpuC expression were determined by Western blotting. OpuCA comparison was ultimately normalized based on total protein concentrations (5 mg ml⁻¹), with 10 µl added. A clear band was detected by Western blotting for purified OpuCA and this band was absent from the ΔopuCA mutant indicating that the antibodies produced were specific for OpuCA (Fig. 2.S2). OpuCA
Fig. 2.3. OpuCA expression is induced in proportion to the osmotic stress. The OpuCA protein was detected in three biological replicates of crude cell extracts by Western blotting using anti-OpuCA-His polyclonal antibodies as described in Fig. 2.S2 legend (supplementary material). Crude cell extracts were prepared from exponential-phase cells (Exp) or stationary-phase cells (Stat) of the wild-type (wt) or the ΔsigB and ΔopuCA mutant strains grown at 37°C in BHI broth over a range of NaCl concentrations (0, 0.3, 0.6, 0.9). Total protein concentrations were normalized to 5 mg ml⁻¹ and 10 μl of each protein extract was loaded. Western blotting was carried out using semi-dry transfer with incubations and washing steps followed by a chemiluminescent light detection as all described in Fig. 2.S2 legend (supplementary material).

Strikingly, the highest level of OpuCA in wild-type occurred with the most severe osmotic challenge, specifically 0.9 M NaCl. The levels of OpuCA were found to increase gradually approximately in proportion to the salt concentration in the medium during exponential growth. These results correlate well with what was observed at the transcriptional level (Fig. 2.2) and suggest that OpuC levels reflect the extent of σB activation during balanced growth. Thus the availability of antibodies against OpuCA should generally prove useful in measuring σB activity in L. monocytogenes. It is worth noting that in stationary phase the levels of OpuCA remained high over the whole range of salt concentrations tested (Fig. 2.2), suggesting that expression was fully induced in stationary phase regardless of whether salt was added or not. This result suggests that σB might be maximally active in stationary phase, presumably because the entire cellular pool of σB is associated with RNA polymerase.
Overall the findings of this study suggest that $\sigma^B$ activation by osmotic stress is proportional to magnitude of the stress, with a wide dynamic range of activities apparent. The kinetics of transcriptional activation in response to osmotic upshock are extremely fast, with a peak of activity occurring 15 minutes after upshock. Together these data show that $\sigma^B$ activation is carefully calibrated to meet the precise conditions encountered, a feature that is likely to contribute to the remarkable adaptability of this pathogen.

We thank our colleagues in the Bacterial Stress Response Group for useful comments and discussions. The $\Delta$sigB strains used in this study were generously supplied by Prof Kathryn Boor (Cornell University) and Dr Cormac Gahan (University College Cork). This work was supported by an Irish Research Council for Science, Engineering and Technology EMBARK together with Thomas Crawford Hayes funding awards to MU and by a Science Foundation Ireland Research Frontiers Programme grant (05/RFP/Gen0044).
copyright
all rights reserved
Fig. 2.S1. Induction of *opuCA*, *lmo2230*, *lmo2085* and *sigB* transcription in response to osmotic stress is $\sigma^B$-dependent. Relative transcript levels of three $\sigma^B$-dependent genes (*opuCA*, *lmo2230*, *lmo2085*) and *sigB* in exponential-phase cultures (OD$_{600} = 0.6$) of the wild-type and the $\Delta$*sigB* mutant, grown in BHI with (+) or without (-) 0.5 M NaCl. RNA extracts and cDNA were prepared as previously described (25). All transcript levels were first normalised to the corresponding 16S RNA levels and then expressed as a percentage of the maximal level of *sigB* transcript detected in the experiment (thereby allowing the relative transcript levels of the 4 genes to be compared). The values presented on the graphs are the means of three independent experiments and error bars indicate the standard deviations (n=3). Numbers shown above the bars indicate statistically significant differences in relative gene expression (fold-change) between the stressed (+NaCl) and non-stressed (-NaCl) conditions ($P<0.05$ in Student’s $t$ test).
Fig. 2.S2. OpuCA expression is σ\textsuperscript{B}-dependent. The OpuCA protein was detected in crude cell extracts by Western blotting using anti-OpuCA-His polyclonal antibodies, which were produced in chickens. Histidine-tagged OpuCA was first synthesized (\textit{lmol428} sequence EMBL accession AL591979.1) and purified by Fusion Antibodies (Belfast). Two leghorn-type chickens (\textit{Gallus gallus domesticus}) were immunised three times with purified His-tagged OpuCA (1.2 mg ml\textsuperscript{-1}) over a nine week period. At each immunisation antigen was injected intramuscularly in four separate sites into the breast muscle in a volume of 150 μl per immunisation site (with 50 μg of antigen in each). The first immunisation consisted of the OpuCA mixed 1:1 by volume with Freunds Complete Adjuvant (Difco), each subsequent immunisation consisted of the OpuCA mixed with an equal volume of Freunds Incomplete Adjuvant (Difco). Seven days after the third immunisation, each chicken was bled of 1 ml from the wing vein to determine serum antibody response. After a specificity test an additional boost for each chicken was performed in week 12. Then a final bleed of 30 ml from each chicken was taken. Blood was allowed to clot and retract at 4°C overnight before the serum was clarified by centrifugation at 2,500 rpm for 20 min. Stocks of polyclonal antibody were stored in aliquots at -80°C. Protein extracts were prepared from mid-exponential phase (Exp) or stationary phase (Stat) cultures of the wild-type EGD (wt), or the Δ\textit{sigB} and Δ\textit{opuCA} mutant strains grown at 37°C in BHI as previously described (2). Total protein concentrations were normalized to 5 mg ml\textsuperscript{-1} and 10 μl of each protein extract was loaded. Ten μg of purified OpuCA was used as a positive control. 3% w/v skim milk in dH\textsubscript{2}O was used for blocking the membrane and for diluting the primary antibody (chicken polyclonal antiserum) 1:2,000, and the secondary antibody (HRP conjugated to goat Anti-Chicken IgY, Promega) 1:20,000. PBS and PBS with 1% (v/v) Tween20 (Promega) was used for the washing steps. The chemiluminescent light, produced due to the HRP enzymatic reaction with a chemiluminescent substrate (SuperSignal® West Pico Chemiluminescent Kit, Pierce), was captured in a dark room using a light sensitive film (Amersham Hyperfilm ECL, GE Healthcare). SDS-PAGE conditions were further standardised for an optimal separation of non-specific bands and washing conditions together with multiple exposures (ranging from 30 s to 180 s) of
film were carried out to obtain an exposure with reduced non-specific binding. The arrow indicates the position of the OpuCA protein, while the other bands represent non specific binding of the antibodies. All findings were reproducible for three biological replicates of protein extractions.
σB-dependent expression under other stimuli was also investigated. A pool of anti-OpuCA polyclonal antibodies was developed in chickens, after commercial purification of the OpuCA protein (Appendix 1). The anti-OpuCA antibodies were used to infer the induction of σB under conditions related to the GI phase of infection (Appendix 2). Possible limitations of the anti-OpuCA-based approach were related to its stability (Appendix 3) and the reporting of σB-independent expression of OpuCA at cold temperatures (Uratna et al., 2012, Submitted Chapter 4). Moreover, the influence of the range of pH values on σB activity was investigated in steady-grown cells of L. monocytogenes EGD-e wild-type and ΔsigB mutant (Appendix 4), while the kinetics of σB activation were measured by qRT-PCR after uphocking with weak acids in cells of L. monocytogenes EGD-e wild-type (Appendix 5).
CHAPTER 3

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

Published by Bioengeneered
3.1. ABSTRACT

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.
3.2. INTRODUCTION

The gram positive bacterium *Listeria monocytogenes* is a facultative intracellular pathogen that is found widely in the environment (reviewed by Barbuddhe and Chakraborty, 2009). 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 (σB) (Milohanic et al., 2003; Rauch et al., 2005, Ollinger et al., 2008). σB is involved in the initiation of transcription by recognizing σB promoter sequences and allowing the expression of a specific set of genes (σB 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 σB plays an important role both during infections and under stressful conditions (reviewed by O’Byrne and Karatzas, 2008). After the full genome sequence of *L. monocytogenes* became available (Glaser et al., 2001) a number of studies identified components of the σB regulon by looking at genes differentially expressed in *L. monocytogenes* wild-type and corresponding sigB mutants using proteomic approaches (Wemekamp-Kamphuis et al., 2004c; Abram et al., 2008c) or gene microarray technology (Raengpradub et al., 2008; Hain et al., 2008; Kazmierczak et al., 2003, Toledo-Arana et al., 2009).

The mechanism responsible for sensing stress that leads to the activation of σB has not been fully characterized in *L. monocytogenes*. However, the signal transduction system controlling σB activity in *Bacillus subtilis*, has been well studied (reviewed by Hecker et al., 2007). A high level of conservation between the sigB operons in *B. subtilis* and *L. monocytogenes* suggests a shared mechanism of post-translational regulation of σB activity. In *B. subtilis* this mechanism involves partner switching of an anti sigma factor called RsbW between σB and anti-anti sigma factor called RsbV; when RsbW is sequestered by RsbV, σB is free to participate in transcription. In order to test this model and to develop an understanding of how σB is controlled in *L. monocytogenes*, molecular tools for measuring the activity status of σB in this pathogen are required. Therefore in the present study we aimed to develop an effective reporter for measuring σB activity in *L. monocytogenes*, which would then
allow important questions to be addressed about the conditions that trigger the activation of $\sigma^B$ 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* (Shin *et al*., 2010a, Riedel *et al*., 2007, Ferreira *et al*., 2003). 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 utilised in a wide range of other organisms including bacteria (Southward and Surette, 2002). As measurement of GFP activity in prokaryotes was reported to be affected reversibly by protein oxidation, the pH value of the medium and temperature (Hansen *et al*., 2001) several chromophore variants of wild-type GFP (wtGFP) have been developed for use as a reporters under a wide range of conditions (Cubitt *et al*., 1995). For a reporter of $\sigma^B$ 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 (Heim *et al*., 1995, Patterson *et al*., 1997). 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 utilisation of EGFP in living cells under less harmful conditions due to lower energies involved in visualisation. EGFP folding and chromophore formation is also faster (Iizuka *et al*., 2012) and its structure was reported to be stable over osmolarity and temperature ranges (Gerena-Lopez *et al*., 2004).

One of the key factors in considering a successful reporter of $\sigma^B$ activity in *L. monocytogenes* is the choice of an effective $\sigma^B$–dependent promoter. In the pioneering *in vitro* studies of sporulation in *B. subtilis* Haldenwang and Losick (1979) used the *ctc* gene in their transcription assays, which led to the discovery of one of the first bacterial alternative sigma factor, $\sigma^B$. Since then *ctc* has become the
first-choice reporter gene for $\sigma^B$ activity studies in B. subtilis, where it has been used to investigate a whole range of different stress stimuli (Benson and Haldenwang, 1993b, Brigulla et al., 2003, Pragai and Harwood, 2002). The ctc gene, described as being positively regulated by $\sigma^B$ in L. monocytogenes (Kazmierczak et al., 2003) was also used in a transcriptomic approach to investigate blue and red light activation of $\sigma^B$ in L. monocytogenes (Ondrusch and Kreft, 2011). The study demonstrated that in a $\Delta\text{sigB}$ mutant there was still substantial ctc transcript levels, suggesting that in L. monocytogenes ctc transcription is not fully dependent on $\sigma^B$. The sigB gene itself is known to be autoregulated (Becker et al., 1998, Rauch et al., 2005) and has previously been used in L. monocytogenes as a reporter for the role of $\sigma^B$ in biofilm formation (van der Veen and Abee). 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 $\sigma^B$-independent manner from the $\sigma^A$ promoter located upstream from rsbR and yielding the transcript for the whole operon including rsbRSTU followed by rsbV, rsbW, sigB and rsbX (Becker et al., 1998) or within a 10 gene operon starting upstream from lmo0887 (Toledo-Arana et al., 2009).

Our recently published work evaluated transcript levels of sigB and other $\sigma^B$-dependent genes as a reporter of $\sigma^B$ activity (Utratna et al., 2011). Out of four $\sigma^B$-dependent genes investigated (sigB, opuCA, lmo2085, lmo2230), expression of lmo2230, which encodes a putative arsenate reductase, was strongly $\sigma^B$-dependent and demonstrated the highest level of induction following osmotic stress. Selected studies on the $\sigma^B$ regulon in various strains of L. monocytogenes over a range of different stress conditions (van der Veen et al., 2007, Hain et al., 2008, Kazmierczak et al., 2003, Chan et al., 2007a, Raengpradub et al., 2008, Chaturongakul and Boor, 2006, Oliver et al., 2009) have confirmed the high induction and strong $\sigma^B$-dependence of lmo2230 expression. These studies also show that the $\sigma^B$-dependent activation of lmo2230 transcription occurs in different strains and in response to different stresses. The $\sigma^B$ promoter sequence of lmo2230 was predicted with Hidden Markov model (HMM)-based searches and its transcriptional start site was confirmed by RACE-PCR (Kazmierczak et al., 2003). lmo2230 (designated as arsC) has been already successfully used among other $\sigma^B$-regulated genes as a reporter of $\sigma^B$ activity after exposure to light (Ondrusch and Kreft, 2011). 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 \textit{lmo2230} to monitor $\sigma^B$-dependent expression that will be quicker and more effective for testing over a wide range of conditions.

3. 3. MATERIALS AND METHODS

3.3.1. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. \textit{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 OD$_{600}$=0.05 to OD$_{600}$=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. \textit{E. 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 μg ml$^{-1}$ for \textit{L. monocytogenes}; Ampicillin (Amp) 100 μg ml$^{-1}$ for \textit{E. coli}. (Smith and Youngman, 1992)

3.3.2. Construction of a \textit{L. monocytogenes} EGD-e $\Delta rsbV$ mutant

\textit{L. monocytogenes} EGD-e $\Delta rsbV$ mutant was constructed using the splicing by overlap extension (SOEing) PCR technique (Horton \textit{et al.}, 1990) 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 \url{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 \textit{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 the \textit{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
Table 3.1. Plasmids and strains used in this study

<table>
<thead>
<tr>
<th>Source or reference</th>
<th>PLASMIDS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pKSV7 (pUC18 and pBD95 integrated shuttle vector for <em>E. coli</em> and <em>L. monocytogenes</em> carrying a temperature sensitive oriC from pE194) (Smith and Youngman, 1992)</td>
</tr>
<tr>
<td></td>
<td>pCR®-XL-TOPO® Invitrogen™</td>
</tr>
<tr>
<td></td>
<td>pEC04 (pCR®-XL-TOPO® containing the ΔrsbV deletion cassette)</td>
</tr>
<tr>
<td></td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>pEC06 (pKSV7 containing ΔrsbV DNA deletion cassette)</td>
</tr>
<tr>
<td></td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>pBluescript II-Plmo2230::egfp Eurofins MWG Operon</td>
</tr>
<tr>
<td></td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>PLmo2230::egfp</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source or reference</th>
<th>STRAINS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Collection number</td>
</tr>
<tr>
<td></td>
<td>E. coli DH5α/pKSV7</td>
</tr>
<tr>
<td></td>
<td>N. Freitag COB082</td>
</tr>
<tr>
<td></td>
<td>One Shot® TO P10 Electrocomp™ E. coli Invitrogen™ COB267</td>
</tr>
<tr>
<td></td>
<td>E. coli TOP10/pBluescript II-Plmo2230::egfp This study COB515</td>
</tr>
<tr>
<td></td>
<td>E. coli TOP10/pKSV7-Plmo2230::egfp This study COB516</td>
</tr>
<tr>
<td></td>
<td>L. monocytogenes EGD-ΔrsbV This study COB517</td>
</tr>
<tr>
<td></td>
<td>L. monocytogenes EGD-ΔrsbVΔsigB This study COB411</td>
</tr>
<tr>
<td></td>
<td>L. monocytogenes EGD-ΔrsbV::pKSV7-Plmo2230::egfp This study COB501</td>
</tr>
<tr>
<td></td>
<td>L. monocytogenes EGD-ΔrsbV/pKSV7-Plmo2230::egfp This study COB477</td>
</tr>
<tr>
<td></td>
<td>L. monocytogenes EGD-ΔrsbV::pKSV7-Plmo2230::egfp This study COB495</td>
</tr>
<tr>
<td></td>
<td>L. monocytogenes EGD-ΔrsbV::pKSV7-Plmo2230::egfp This study COB491</td>
</tr>
</tbody>
</table>

Table 3.2. Primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′ to 3′)</th>
<th>Anneal (°C)</th>
<th>Collection number</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13-F</td>
<td>CAGGAAACAGCTATGAC</td>
<td>50</td>
<td>COB162</td>
</tr>
<tr>
<td>M13-R</td>
<td>GTAAAACGACGGCCAG</td>
<td>50</td>
<td>COB163</td>
</tr>
<tr>
<td>rsbV-CACC-F</td>
<td>CACCACTGAATATTAGTATAGAAATAAA</td>
<td>55</td>
<td>COB328</td>
</tr>
<tr>
<td>rsbV-his6-R</td>
<td>TCAATGATGATGATGATGTGCATT</td>
<td>55</td>
<td>COB329</td>
</tr>
</tbody>
</table>

Copyright © all rights reserved
3.3.3. Design and construction of $P_{lmo2230}::egfp$ gene fusion strains

The reporter plasmid pKSV7-$P_{lmo2230}::egfp$ (Fig. 3.1A) was designed to contain a fusion of the $\sigma^B$-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 GENEdius software) $egfp$ (accession AY192024.1), the gene coding for enhanced green fluorescent protein (EGFP). (Heim et al., 1995) First, $egfp$ was synthesised together with 443 bp sequence of the $lmo2230$ promoter region upstream from its start codon (Kazmierczak et al., 2003) flanked by BamHI and EcoRI restriction sites (together this gene fusion construct was designated $P_{lmo2230}::egfp$). The gene fusion was synthesised by Eurofins MWG and inserted into pBluescriptII yielding pBluescriptII-$P_{lmo2230}::egfp$. The $P_{lmo2230}::egfp$ fragment including the BamHI and EcoRI 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 BamHI and
EcoRI 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, \( \Delta \text{sigB} \) and \( \Delta \text{rsbV} \) derivative mutant strains as described earlier.\cite{Abram2008a} The transformants were selected on BHI agar plates containing 10 \( \mu \)g ml\(^{-1}\) of chloramphenicol, kept for 48 h at 30°C.

**Fig. 3.1.** Design of the reporter plasmid pKSV7-P_{lmo2230}::egfp containing a fusion of the \( \sigma^B \)-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 (443bp) after incubation at 42°C (non-permissive for autonomous plasmid maintenance in \( L. \) monocytogenes) in the presence of chloramphenicol selection.

### 3.3.4. Chromosomal integration of the P_{lmo2230}::egfp reporter fusion

Homologous recombination was utilized to construct recombinant strains of \( L. \) monocytogenes EGD-e wild type, \( \Delta \text{sigB} \) and \( \Delta \text{rsbV} \) derivative mutants carrying single copies of the P_{lmo2230}::egfp reporter fusion integrated into the chromosome (Fig. 3.1B). 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 hours at 42°C. At a non-permissive temperature
the vector was unable to replicate autonomously and integrated into the $P_{lmo2230}$ 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-$P_{lmo2230}$::egfp plasmid and EGD-e genomic DNA (Table 2).

3.3.5. Microscopic quantification of EGFP fluorescence

To visualise fluorescence due to $\sigma^B$-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 $\mu$m pore, 10 mm diameter, black polycarbonate membrane filters (Millipore). For preliminary analyses, 1 ml of bacterial cells was centrifuged, resuspended in 50 $\mu$l of sterile PBS and 5 $\mu$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 s in a dark room using the B-2A filter covering GFP wavelengths together with 1/8 Neutral Density filter (ND8) for minimising EGFP photobleaching. Relative fluorescence intensities and detectable cell counts were reported after automated image processing of multiple fields with ImageJ 1.44 software (Collins, 2007) with appropriate manipulations as described by others (Hamilton, 2009, Selinummi et al., 2005). For each experimental condition a minimum of three biological replicates were analysed and ten randomly selected fields were captured.

3.3.6. Western blot analyses

Proteins were extracted using a sonication-based method (Abram et al., 2008a) 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 $\mu$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).

3.3.7. 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 -20ºC. 1 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 μl 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 μL/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 dot plot side scatter and forward scatter values for two biological and three technical replicates.

3.4. RESULTS

3.4.1. 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 synthesised 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 ΔsigB and ΔrsbV 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 harbouring the pKSV7-P\textsubscript{lmo2230::egfp} plasmid were brightly fluorescent, although considerable heterogeneity in the level of EGFP fluorescence could be observed in the population (Fig. 3.2). Analysis of randomly selected microscopic fields with phase contrast and fluorescence microscopy suggests that some portion of wild-type cells transformed with pKSV7-P\textsubscript{lmo2230::egfp} were non-fluorescent or might express EGFP at a level not detectable with this method. In contrast to the wild-type, the Δ\textit{sigB} mutant strain carrying pKSV7-P\textsubscript{lmo2230::egfp} showed no fluorescence, suggesting that σ\textsubscript{B} is necessary for activation of \textit{egfp} expression from the \textit{lmo2230} promoter. Furthermore, the Δ\textit{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\textsubscript{B} remains permanently sequestered by RsbW and is therefore unavailable to drive transcription from σ\textsubscript{B} promoters.

Fig. 3.2. Fluorescence of \textit{egfp}-containing cells of \textit{L. monocytogenes} EGD-e wild type, Δ\textit{sigB} and Δ\textit{rsbV} mutants after transformation with pKSV7-P\textsubscript{lmo2230::egfp}. Phase-contrast and fluorescence microscopy of corresponding fields were performed after 1 ml of stationary phase culture was centrifuged, resuspended in 50 μl of sterile PBS and 5 μl 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.
Fig. 3.3. EGFP is expressed of *L. monocytogenes* EGD-e wild type but not in ΔsigB and ΔrsbV backgrounds. Western blot analyses were performed using protein extracts from stationary phase cultures (normalized to 5 mg ml⁻¹ total protein concentrations and 10 µl of these samples separated by SDS-PAGE) with chicken polyclonal anti-GFP antibody (Abcam) HRP-conjugated goat Anti-Chicken antibody (Promega) and chemiluminescent detection.

To check if the lack of fluorescence of ΔsigB, and ΔrsbV 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, ΔsigB, and ΔrsbV 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 ΔsigB (Fig. 3.3A), and the ΔrsbV strains (Fig. 3.3B). These findings confirmed σB-dependent expression of EGFP and also indicated an absence of RsbV-independent activation of σB under the conditions investigated.
3.4.2. Quantification of *lmo2230*-promoter-driven expression with fluorescent microscopy

In order to eliminate any artefacts 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. 3.1A) was integrated into the genomes of *L. monocytogenes* EGD-e wild type, Δ*sigB* and Δ*rsbV* strains by homologous recombination (Fig. 3.1B). 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 Δ*sigB* and Δ*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. 3.4). 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. 3.4B) and subsequently determining the mean fluorescent intensity of the objects detected (Fig. 3.4C). 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 (Gerena-Lopez et al., 2004). As expected EGFP expression was increased by salt in exponential phase and a higher fluorescence was observed in stationary phase when compared to the exponential phase of growth (Fig. 3.4). The salt induction in exponential phase could be visually observed in randomly selected microscopic fields (Fig. 3.4A; n≥30) and was further demonstrated by quantifying the number of fluorescent objects detected (Fig. 3.4B) and their relative fluorescence (Fig. 3.4C). However, the activation of σ^B^ by salt was not found to be statistically significant in stationary phase (Fig. 3.4C). 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.
Fig. 3.4. $\sigma^B$-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 $\mu$m pore polycarbonate membrane (Millipore) by filtration. For each experimental condition three biological replicates were analysed 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 s 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 (Collins, 2007, Hamilton, 2009).

3.4.3. Activation of $\sigma^B$ is rapid after osmotic upshock

To determine whether our reporter system is able to detect quick changes of $\sigma^B$ activity reported by earlier studies (Utratna *et al.*, 2011; van der Veen *et al.*, 2007), 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. 3.5B). The reporter approach demonstrated an induction of $\sigma^B$ 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 $\sigma^B$ following osmotic upshock.

Fig. 3.5. $\sigma^B$-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 $\mu$m pore polycarbonate membrane (Millipore) by filtration. For each experimental condition three biological replicates were analysed 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 (Collins, 2007, Hamilton, 2009).
3.4.4. Flow cytometry reveals heterogeneous activation of $\sigma^B$ 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 analysed whereas other methods give average data for the whole population. When wild-type cells without a reporter fusion were analysed 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 analysed, following aerobic growth to exponential phase ($OD_{600}=0.6$) at $37^\circ C$, 27.8% of the particles detected had fluorescence levels that fell within the defined gate (Fig. 3.6). When a FCM analysis was performed on a culture of the $\Delta\sigma_{B}$ mutant grown under the same conditions, no significant fluorescence was detected above the background level, indicating that $\sigma^B$ 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 $\Delta\sigma_{B}$ mutant (Fig. 3.6). 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 $\sigma^B$ activity being proportional to the extent of NaCl stress (Utratna et al., 2011).
Fig. 3.6. 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-Plmo2230::egfp (WT-egfp) and ΔsigB-P_lmo2230::egfp (Δσ^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.
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. 3.6). 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 $\sigma^B$-dependent (Fig. 3.6). This result indicates that there is some overlap between cellular autofluorescence and EGFP fluorescence and also shows that the range of $\sigma^B$ activities within the population is considerable. The heterogeneity of $\sigma^B$ activities within the population exposed to hyperosmotic stress raises new and interesting questions about how individual cells sense and respond to osmotic stress.

### 3.5. DISCUSSION

In the present study we developed a rapid EGFP-based reporter assay for measuring $\sigma^B$ activity in *L. monocytogenes*. The system was carefully designed based on available literature to have the strong $\sigma^B$-dependent promoter of *lmo2230*, which ensured that cells were highly fluorescent following the activation of $\sigma^B$. The reporter approach showed increased $\sigma^B$ activity in stationary phase and during hyperosmotic stress, which was applied either continuously or suddenly. Furthermore, an analysis by flow cytometry revealed that $\sigma^B$ activation was heterogeneous in the population and proportional to the extent of stress. The $\sigma^B$-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 $\sigma^B$ 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 minutes of heat stress (van der Veen *et al.*, 2007) and also 15 minutes after osmotic upshock when more than 160-fold higher levels of *lmo2230* in wild-type were reported comparing to unstressed cells at time zero (Utratna *et al.*, 2011). Taken together these findings give an opportunity
for monitoring activation of $\sigma^B$ 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 $\sigma^B$ 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) (Andersen et al., 1998) makes it impossible to observe a drop in $\sigma^B$ activity after the removal of stress, while this decrease can be demonstrated by monitoring the transcript levels of $lmo2230$ and other $\sigma^B$-regulated genes under similar conditions (Utratna et al., 2011) and after heat shock (van der Veen et al., 2007). Future versions of this reporter could include less stable GFP variants (with $ssrA$ RNA tags recognized by housekeeping proteases) (Andersen et al., 1998) in order to overcome this limitation. When the time scale for $\sigma^B$ activation was compared between the RNA based approach described earlier (Utratna et al., 2011) and the $P_{lmo2230}$::$egfp$ reporter (Fig. 3.5B), the maximal $\sigma^B$ activation was evident approximately 15 minutes 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 (Cubitt et al., 1995, Sniegowski et al., 2005). 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 $\sigma^B$ activity in response to stress.

Another unique advantage of the $P_{lmo2230}$::$egfp$ reporter is that $\sigma^B$ activation can be studied both at the single cell and the population levels, using microscopy and flow cytometry. Using 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. 3.6). This result indicates that $\sigma^B$ 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 (Elowitz et al., 2002). It will be interesting in future
studies to determine whether cell-cell differences in $\sigma^B$ activation influences the survival of individual cells in foods or within the gastrointestinal tract (Booth, 2002).

In an $\Delta rsbV$ background no EGFP expression was detected from the $P_{lmo2230::egfp}$ reporter (Fig. 3.2 & 3.3B), consistent with the current model for $\sigma^B$ activation, which proposes that RsbV acts as an anti-anti-sigma factor that facilitates $\sigma^B$ activation when it interacts with the anti-sigma factor RsbW (Hecker et al., 2007). 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 $\sigma^B$ activation (Chaturongakul and Boor, 2004). This highlights the potential use of the $P_{lmo2230::egfp}$ reporter in conducting studies on the regulation of $\sigma^B$. 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 homologues 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 $\sigma^B$ activation.

In summary, the $P_{lmo2230::egfp}$ fusion was developed and shown to be a reliable indicator of $\sigma^B$ activity in $L. monocytogenes$, both during different phases of growth and under conditions of hyperosmotic stress. The tight $\sigma^B$-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.
3.6. ACKNOWLEDGEMENTS

We thank our colleagues in Microbiology and the Bacterial Stress Response Group for useful discussions. We thank N. Freitag for supplying plasmid pKSV7. This work was supported by a Science Foundation Ireland Research Frontiers Programme grant (05/RFP/GEN0044) and by Irish Research Council for Science, Engineering and Technology EMBARK funding together with Thomas Crawford Hayes award to MU. CB and RC were supported by Science Foundation Ireland grant SRC 09/SRC/B1794 and by a Science Foundation Ireland Stokes Professorship to RC.
Appendices (6-16) related to Chapter 3

The development of the pKSV7-P\textsubscript{lmo2230::egfp} reporter vector first involved a commercial synthesis of the reporter fusion insert (P\textsubscript{lmo2230::egfp}), followed by a series of cloning steps (Appendix 6). The reporter vector was transformed into \textit{L. monocytogenes} EGD-e wild-type, Δ\textit{sigB} and Δ\textit{rsbV} strains and integrated into their chromosomes by homologous recombination, to obtain single copy of the reporter in each cell. Transformations and integrations, as well as their stability, were confirmed by analysis of PCR products carried out with selected pairs of primers (Appendix 7). To measure levels of fluorescence exhibited by \textit{L. monocytogenes} EGD-e wild-type carrying a single copy of the \textit{egfp} gene expressed from P\textsubscript{lmo2230}, quantitative fluorescence microscopy was optimised by taking into account appropriate sample preparation, image acquisition and digital data analysis (Appendix 8). The optimisation by a series of control experiments resulted in a protocol for cell fixation and filtration, giving a diluted and spread-out layer of bacterial cells, with their fluorescence preserved (Appendix 9). The fluorescence of cells could be captured at a fixed exposure time, with the B2A filter covering GFP spectra in the fluorescent microscope (Nikon Eclipse E600), coupled with aCCD camera. The images obtained were analysed further with ImageJ software, to calculate the fluorescence intensity of cells after background subtraction (Appendix 10), as well as number of particles exhibiting fluorescence in each field being captured (Appendix 11). Thus, a proportion of the fluorescent population could be calculated by a comparison of particles captured by phase contrast and fluorescence microscopy of corresponding fields (Appendix 12).

Heterogeneity of σ\textsuperscript{B} activation within \textit{L. monocytogenes} EGD-e wild-type population grown under identical experimental conditions was observed with microscopy, and was further measured by a more automated approach, with flow cytometry. Optimisation of the flow cytometry procedure and the determining of the fluorescence range (gate) characteristic for σ\textsuperscript{B}-driven expression of \textit{egfp} were carried out with a BD Accuri C6 flow cytometer (Accuri Cytometers, Inc.) and BD CFlow software, using WT-\textit{egfp} (positive) and Δ\textit{sigB-egfp} (negative) strains (Appendix 13). Heterogeneity of σ\textsuperscript{B} activation under a range of osmotic stress was first investigated in cells grown in the presence of 0, 0.3 M, 0.6 M and 0.9 M NaCl, up to OD\textsubscript{600}=0.4 (Appendix 14) and, later, after treatment with a range of antimicrobial agents.
Furthermore, the pKSV7-Pmo2230::egfp reporter vector was transformed into \textit{L. monocytogenes} 10403S wild-type, Δ\textit{sigB} and Δ\textit{rsbV}, and integrated into their chromosomes. Thus, single-copy reporter strains were developed in three \textit{L. monocytogenes} 10403S strains for further analysis of σ\textsuperscript{B} activation in different genetic backgrounds and addressing various aspects concerning the strain specificity of the σ\textsuperscript{B}-dependent response (Appendix 16).
CHAPTER 4

Effects of growth phase and temperature on $\sigma^B$ activity within a *Listeria monocytogenes* population: Evidence for RsbV-independent activation of $\sigma^B$ at refrigeration temperatures

Submitted to Foodborne Pathogens and Disease
4.1. ABSTRACT

The alternative sigma factor $\sigma^B$ of *Listeria monocytogenes* is responsible for regulating the transcription of many of the genes necessary for adaptation to both food-related stresses and to conditions found within the gastrointestinal tract of the host. The present study sought to investigate the influence of growth phase and temperature on the activation of $\sigma^B$ in *L. monocytogenes*. Since neither the levels of $\sigma^B$ nor the levels of the OpuCA protein, whose expression is partially under $\sigma^B$ control, proved to be reliable indicators of $\sigma^B$ activity, a reporter fusion that couples expression of enhanced green fluorescent protein (EGFP) to the strongly $\sigma^B$-dependent promoter of *lmo2230* was utilised to monitor $\sigma^B$ activity. Activation of $\sigma^B$ was studied within populations of *L. monocytogenes* EGD-e wild-type, ΔsigB and ΔrsbV throughout growth at both 4°C and 37°C. A similar $\sigma^B$ activation pattern within the population was observed in wt-egfp at 4°C and at 37°C, with the highest induction of $\sigma^B$ occurring in the early exponential phase of growth when the fluorescent population rapidly increased, eventually reaching a maximum in early stationary phase. During balanced growth in BHI induction of $\sigma^B$ activity was heterogeneous, with only a proportion of the cells in the wt-egfp population producing fluorescence levels that were detectable by this reporter system (i.e. above the background autofluorescence level). Moreover, significant RsbV-independent activation of $\sigma^B$ was observed during growth at 4°C, using flow cytometry, microscopy, and Western blotting to determine the induction of EGFP expression from the reporter. This result suggests that an alternative route to $\sigma^B$ activation exists in the absence of RsbV, a finding that is not explained by the current model for $\sigma^B$ regulation. Taken together these data suggest that activation of $\sigma^B$ occurs heterogeneously and is modulated by growth phase, but there is little evidence of temperature-dependent modulation.
4.2. INTRODUCTION

The ability of *Listeria monocytogenes* to cause foodborne listeriosis depends on a multi-faceted stress response that is activated under conditions related to food processing including high concentrations of salt, acidic pH, limited oxygen, antimicrobial agents and a wide range of temperatures from -0.4 to 45 °C (Gandhi and Chikindas, 2007). The remarkable adaptability of this bacterium is partly modulated by transcriptional regulators that tailor gene expression to the conditions encountered, with a pivotal role being played by sigma factors that target RNA polymerase to specific promoter sequences (Chaturongakul et al., 2008). The alternative sigma factor, sigma B (σ^B^), first identified in *L. monocytogenes* based on homology to the general stress response sigma factor from the non-pathogenic bacterium *Bacillus subtilis* (Becker et al., 1998, Wiedmann et al., 1998) coordinates the response to a range of stresses as evidenced by the pleiotropic phenotypes associated with a *sigB* deletion (reviewed by O'Byrne and Karatzas, 2008). A core set of σ^B^-dependent genes (σ^B^ regulon) has been described in *L. monocytogenes* and shown to be upregulated in response to a range of conditions including osmotic stress, cold shock, heat shock, acid stress, during stationary phase of growth and under conditions encountered in gastrointestinal tract (Abram et al., 2008b, Kazmierczak et al., 2003, Becker et al., 1998, van der Veen et al., 2007, Toledo-Arana et al., 2009). There is significant overlap between the PrfA virulence regulon and the σ^B^ regulon (Milohanic et al., 2003) with evidence that σ^B^ may even modulate PrfA activity at the intracellular stage of infection (Ollinger et al., 2009). Thus σ^B^ plays important roles in both virulence and in the general response to stress, which makes understanding its regulation essential for future strategies aimed at controlling this pathogen in the food chain and within the host.

The current model of σ^B^ activation in *L. monocytogenes* is based on the high level of similarity of the sigB operon to that from *B. subtilis* (rsbR-rsbS-rsbT-rsbU-rsbV-rsbW-sigB-rsbX) and it suggests post-translational regulation of σ^B^ activity together with σ^B^ autoregulation at the transcriptional level (Hecker et al., 2007). In the absence of stress stimuli σ^B^ interacts with an anti-sigma factor, RsbW, which renders it unavailable for interaction with RNA polymerase. The dephosphorylation of the
anti-anti sigma factor RsbV, which occurs in response to stress, is catalysed by the protein phosphatase RsbU. This renders RsbV capable of interacting with RsbW, which in turn liberates $\sigma^B$, allowing it to participate in transcription. RsbU activity is in turn regulated through an interaction with RsbT, whose availability is determined by its association with a high molecular weight (~2 MDa) stress sensing complex called a “stressosome”. In *B. subtilis* environmental (physical and chemical) stresses influence $\sigma^B$ activity in this way but energy-related stresses are transduced by an alternative phosphatase called RsbP. Since no homologue of RsbP is encoded in the genome of *L. monocytogenes*, both environmental and energy stresses are proposed to be transmitted through RsbU (Shin *et al.*, 2010a, Chaturongakul and Boor, 2004), but in both organisms the initial stress sensing mechanism remains to be elucidated.

Several studies have investigated the role of $\sigma^B$ in allowing *L. monocytogenes* to grow at low temperatures. However studies addressing the effects of a *sigB* deletion on the phenotypic characteristics of *L. monocytogenes* at low temperature have reported conflicting observations. A $\Delta$*sigB* strain of *L. monocytogenes* 10403S had reduced growth in a defined medium (DM) at 8°C (Becker *et al.*, 1998) and *L. monocytogenes* EGD-e $\Delta$*sigB* was reported sensitive to freeze-thaw cycles (Wemekamp-Kamphuis *et al.*, 2004c). In contrast, a $\Delta$*sigB* derivative of *L. monocytogenes* EGD did not show impaired growth in DM at 3°C (Brondsted *et al.*, 2003) and *L. monocytogenes* 10403S $\Delta$*sigB* had a similar growth pattern to the wild-type when grown at 4°C in BHI over 12 days (Chan *et al.*, 2007a, Chan *et al.*, 2008). The available evidence is also unclear on the question of whether of $\sigma^B$ activity is elevated during growth at low temperatures. Transcription of the autoregulated *sigB* operon is induced at cold temperatures suggesting that $\sigma^B$ activity is elevated during low temperature growth (Becker *et al.*, 1998, Becker *et al.*, 2000). The promoter of the *opuC* operon, which encodes a compatible solute uptake system known to be regulated by $\sigma^B$ (Fraser *et al.*, 2003, Fraser *et al.*, 2000, Utratna *et al.*, 2011, Chapter 2), has also been used to look at $\sigma^B$ activity at low temperatures. One study reported that *opuCA* transcript levels are unaffected during temperature downshift or growth at 4°C (Chan *et al.*, 2007a), while another study observed that *opuCA* transcription is induced after temperature downshift (Shin *et al.*, 2010a). However, the presence of a $\sigma^A$–dependent promoter upstream from *opuCA* makes interpretation of these results more difficult (Chan *et al.*, 2007a). Thus the uncertainty in the literature regarding
the role and regulation of σ\textsuperscript{B} during cold adaptation made it important to investigate this question further.

To clarify the role and activity of σ\textsuperscript{B} during adaptation to low temperature growth we have monitored σ\textsuperscript{B} activity during prolonged growth at 4°C in comparison to a culture growing at 37°C. σ\textsuperscript{B} activity was monitored by measuring the expression of two genes known to be under direct σ\textsuperscript{B} control, \textit{opuCA} and \textit{lmo2230}, which encodes a putative arsenate reductase (Glaser \textit{et al}., 2001, Fraser \textit{et al}., 2003). Polyclonal antibodies were used to detect the OpuCA protein while \textit{lmo2230} expression was monitored using an EGFP (enhanced green fluorescent protein) reporter fusion to the strongly σ\textsuperscript{B}-dependent promoter of the \textit{lmo2230} gene (Utratna \textit{et al}., 2011, Chapter 2, Utratna \textit{et al}., 2012, Chapter 3). Fluorescence measurements were made using flow cytometry throughout growth in wild-type, ΔsigB and ΔrsbV backgrounds. The measurements revealed heterogeneous activation of σ\textsuperscript{B} within growing populations of cells, with increased activation evident as cells progressed through exponential phase, reaching maximum activation in stationary phase. Similar results were observed at both growth temperatures suggesting that σ\textsuperscript{B} activity was not increased by reduced temperatures \textit{per se}. The study provides important new insights into the temporal and population-related parameters that modulate the activity of sigma B in \textit{L. monocytogenes}.

4.3. MATERIALS AND METHODS

4.3.1. Growth conditions
\textit{L. monocytogenes} EGD-e strains used in this study are listed in Table 1. For growth and flow cytometry (FCM) experiments at 37°C overnight cultures (16 h) were inoculated into 25 ml of sterile Brain Heart Infusion (BHI) broth (LabM) in 250 ml flasks to give a starting OD\textsubscript{600}=0.05. OD\textsubscript{600} readings were taken from rotary (180 rpm) shaken cultures at 45 min intervals over 7 hours and samples of cells were taken for FCM and fixed as previously described (Utratna \textit{et al}., 2012, Chapter 3). Growth at 4°C was also monitored from a starting OD\textsubscript{600}=0.05. Stationary culture cells grown in BHI at 37°C were inoculated into 200 ml of sterile BHI in 2L flasks and gently agitated (30 rpm) on the rocker (Stuart See-Saw Rocker SSL4) at 4°C.
Samples were taken daily over 12 days for monitoring OD$_{600}$ and at 3 days intervals for FCM.

Table 4.1. Plasmids and strains used in this study
4.3.2. Protein extraction
For determining levels of σ^B bacterial cultures were grown in 50 ml of BHI for 3 h at 37°C. Then 1 ml of culture was removed at 60 min intervals into a tube containing chloramphenicol at a final concentration of 10 μg ml\(^{-1}\), to prevent further protein translation during the sample preparation steps. Samples were stored on ice until the completion of the experiment. Each 1 ml culture was then centrifuged at 12,000 g for 10 min to pellet bacterial cells. The supernatant was discarded and each cell pellet was resuspended in 100 μl of BugBuster™ cell lysis reagent (Novagen, USA) containing 1% (v/v) DNaseI, 1% (v/v) Halt Protease Inhibitor Cocktail, and 1% (w/v) Lysozyme (Sigma, USA). Each cell suspension was then incubated at 37°C for 1 h with agitation. The resulting cell lysates were centrifuged at 5,000 g for 10 min to remove insoluble material. For OpuCA levels large-scale protein extraction was carried out by growing bacterial cultures at 37°C with a volume of 500 ml and removing 50 ml of cultures at 50 min intervals into a tube containing chloramphenicol at a final concentration of 10 μg ml\(^{-1}\). A disruption of cells was accomplished by sonication and protein extraction was performed as previously described (Abram et al., 2008b). For EGFP and RsbW levels proteins were extracted from 100 ml of stationary cultures grown for 12 days at 4°C using the sonication-based method with a slight modification; cells were fixed in 1:1 volume of ice cold 1:1 (v/v) methanol/ethanol mixture for 10 min at -20°C before centrifugation. The concentrations of protein extracts were determined by the RC DC Protein Assay Kit (BioRad). The clarified protein extracts were stored at -20°C until required.

4.3.3. Polyclonal antisera generation
Genes corresponding to rsbW and sigB were cloned from L. monocytogenes EGD-e using primers listed in Table 2 and transformed into E. coli BL21 (DE3). Each genetic construct was cloned into pET101D with T7 promoter to include a 3’ polyhistidine tag for downstream purification requirements, yielding pEC02 and pEC03. Cultures of E. coli harbouring either rsbW-his6 or sigB-his6 were grown in LB broth supplemented with 100 μg ml\(^{-1}\) ampicillin and induced for overexpression by addition of 1 mM IPTG at approximately OD\(_{600}\) of 0.5. Cells were grown for 4 h following induction and collected by centrifugation at 10,000 rpm, washed with sterile media and lysed with Bugbuster (Novagen, USA) containing 0.1% (v/v)
DNaseI. The bacterial cell lysate was partitioned between soluble and insoluble material by centrifugation at 10,000 rpm for 10 min. Recombinant proteins present as inclusion bodies were purified from the insoluble fraction by Ni-NTA affinity chromatography using buffers supplemented with 8 M urea. RsbW-His6 and SigB-His6 were subsequently purified and prepared as 2 mg ml$^{-1}$ stocks for immunization carried out by Fusion Antibodies (Belfast). For each protein, two NZW rabbits were injected at several sites on each animal with 1 ml of each recombinant protein stock solution supplemented with Freund’s adjuvant. At 35 days following boosting, serum from individual rabbits was tested for specificity against each target antigen. Approximately 5 months post-immunization, antisera against each antigen was collected. The rabbit anti-RsbW and anti-SigB IgG from each rabbit serum was isolated using protein A chromatography. Each IgG preparation was tested for specificity against target antigens using Western blotting.

### Table 4.2. Primers used in this study

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Primer Name</th>
<th>Primer Sequence (5′ to 3′ Direction)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>COB330</td>
<td>rsbW-Fwd</td>
<td>CACCATGGCAACAATGCATGACCAAAATTAC</td>
<td>This study</td>
</tr>
<tr>
<td>COB331</td>
<td>rsbW-his6 Rev</td>
<td>TCAGTGGTGGTGGATGATGAGGGTTGAGATACTTTTGGC</td>
<td>This study</td>
</tr>
<tr>
<td>COB332</td>
<td>sigB-Fwd</td>
<td>CACCATGCCAAAAGTATCTCAGCTCTG</td>
<td>This study</td>
</tr>
<tr>
<td>COB333</td>
<td>sigB-his6 Rev</td>
<td>TTAATGGTGATGGTGATGGTGCTCCACTTCCTCATTCTG</td>
<td>This study</td>
</tr>
</tbody>
</table>

### 4.3.4. Western blotting

Western blotting analyses were performed using polyclonal antisera (1°Ab) developed in rabbits against σ$^B$ and RsbW (this study) or in chickens against OpuCA (Utratna et al., 2011, Chapter 2) and against GFP (Abcam) with commercial secondary antibodies (2°Ab) HRP-conjugated anti-rabbit or anti-chicken (Promega) at appropriate dilutions (Table 3) in 3% w/v skim milk. Protein extracts were normalized to 5 mg ml$^{-1}$ total protein concentrations and 10 µl of these samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked for 60 min in 3% (w/v) skim milk at room temperature and incubated overnight at 4°C with an appropriate 1°Ab. Incubations with 2°Ab were performed at
room temperature for 60 min. Three 10 min long washing steps with Tween20 (Promega) diluted in PBS (Table 3) followed by 10 min in PBS were performed after each incubation with 1°Ab and 2°Ab. Blots were viewed with a chemiluminescent substrate (SuperSignal® West Pico Chemiluminescent Kit, Pierce) using a light sensitive film (Amersham Hyperfilm ECL, GE Healthcare) or FluorChem Imager (Alpha Innotech Corp) and FluorChem IS-8900 software.

Table 4.3. Antibodies used in this study.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Dilution</th>
<th>Tween20 (v/v)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1°Ab Polyclonal Rabbit Anti-SigB</td>
<td>1/2,000</td>
<td>0.5 %</td>
<td>This study</td>
</tr>
<tr>
<td>1°Ab Polyclonal Rabbit Anti-RsbW</td>
<td>1/1,000</td>
<td>0.1 %</td>
<td>This study</td>
</tr>
<tr>
<td>1°Ab Polyclonal Chicken Anti-OpuCA</td>
<td>1/2,000</td>
<td>1.0%</td>
<td>Utratna et al., 2011</td>
</tr>
<tr>
<td>1°Ab Polyclonal Chicken Anti-GFP</td>
<td>1/2,000</td>
<td>0.5 %</td>
<td>Abcam</td>
</tr>
<tr>
<td>2°Ab HRP- conjugated Goat Anti-Rabbit</td>
<td>1:20,000</td>
<td>0.5 % or 0.1 %</td>
<td>Promega</td>
</tr>
<tr>
<td>2°Ab HRP- conjugated Goat Anti-Chicken</td>
<td>1:20,000</td>
<td>1.0 % or 0.5 %</td>
<td>Promega</td>
</tr>
</tbody>
</table>

4.3.5. Flow cytometry

FCM compared fluorescence of the fusion strains (wt-egfp, ΔsigB-egfp, ΔrsbV-egfp) to one another and the parent strains (wt, ΔsigB, ΔrsbV) were used to determine the level of autofluorescence and to define the EGFP gate above it. Analyses were performed with a BD Accuri C6 flow cytometer (Accuri Cytometers, Inc.) on 100 μl of the fixed and PBS-suspended cells from 96 round bottom plates (Sarstedt). The 488 nm blue laser excitation, FL1 533 / 30 nm (e.g. FITC/GFP) emission channel and 66 μL/min flow rate were used. Each sample was performed in biological triplicate with duplicate analyses in each replication. A minimum of 100,000 events
for each sample were recorded and processed with BD CFlow® Software to determine % of fluorescent population and mean fluorescence values.

4.3.6. Visualization of the fluorescence with microscopy
1 ml aliquots of bacterial cells were harvested with benchtop centrifuge at room temperature, and subsequently resuspended in 50 μl of sterile PBS. 5 μl of the suspension was smeared on microscope slides for visualization with Nikon Eclipse E600 microscope with a CCD camera attached using the B-2A filter and 1/8 Neutral Density (ND8) filter set. Images were recorded and processed for publication with ImageJ 1.44 software.

4.4. RESULTS

4.4.1. σB activity is not correlated with cellular levels of σB protein
Stationary phase is known to induce σB-dependent gene expression (Ferreira et al., 2001, Becker et al., 1998) and sigB itself is known to be autoregulated at the transcriptional level. To determine if the levels of σB changed during growth at 37°C Western blotting was performed on protein extracts taken at 60 min intervals from early exponential to stationary phase using anti-σB polyclonal antibodies. Surprisingly, the highest levels of σB were observed in the exponential phase of growth and σB levels decreased when the cells entered stationary phase (Fig. 4.1A). However, the model for σB regulation established in B. subtilis suggests that σB activity in L. monocytogenes is modulated primarily at the posttranslational level by a partner switching mechanism (Kang et al., 1996). Thus availability of σB rather than σB levels determines its involvement in transcription. To determine when σB was active during growth in L. monocytogenes the expression of the σB-dependent gene opuCA was measured at protein level. OpuCA levels were shown to be significantly lower in exponential phase of growth than observed in stationary phase in L. monocytogenes EGD-e grown at 37°C (Fig. 4.1A). Furthermore, OpuCA was not detectable in the ΔsigB background at any point of growth confirming σB-dependent expression of opuCA under the conditions tested. During growth at 4°C
OpuCA levels were measured in exponential phase and compared to the corresponding extracts from 37°C growth. OpuCA levels were increased at 4°C in comparison to 37°C (Fig. 4.1B). However, significant levels of OpuCA were detected in the ΔsigB background suggesting that opuCA is expressed, at least partly, in a σB-independent manner at 4°C, a finding that is consistent with an earlier transcriptomic study (Chan et al., 2007a). Thus OpuCA levels are not a reliable indicator of σB activity during growth at refrigeration temperatures.

Fig. 4.1 Levels of σB and OpuCA at selected points of growth at 37°C and 4°C. (A) σB levels were determined with Western blotting and rabbit polyclonal anti-σB antibodies in L. monocytogenes wild-type grown in BHI at 37°C at 60 min intervals from mid-exponential to stationary phase. OpuCA levels were monitored with Western blotting and chicken polyclonal anti-OpuCA antibodies in L. monocytogenes wild-type and ΔsigB grown in BHI at 37°C at 50 min intervals from mid-exponential to stationary phase. (B) Levels of OpuCA were determined in L. monocytogenes wild-type, ΔsigB and ΔopuCA grown in BHI up to exponential phase (OD600=0.6) at 4°C in comparison to 37°C.
4.4.2. σ^B is activated during exponential growth at both 37°C and 4°C

To develop an understanding of how the activity of σ^B changes during growth and to investigate an effect of cold temperature on σ^B activity, changes in lmo2230-promoter-driven fluorescence of egfp-tagged strains were analysed using flow cytometry at 4°C and at 37°C, at intervals of three days and 45 min, respectively (Fig. 4.2A & 2B). As expected SGR values for fusion strains grown at 4°C were much lower than those recorded during growth at 37°C (Fig. 4.2C).

Flow cytometry of wt-egfp cells grown at 4°C showed that the fluorescent population increased during the exponential phase of growth from 9.2% to 15.1% in day 3 and 6, respectively (Fig. 4.3A). The highest proportion of fluorescent cells was observed in early stationary phase, reaching 36.6% of the population. The level of EGFP-expressing cells within late stationary phase cultures dropped to 24.7% suggesting that σ^B activity decreased slightly at 4°C during stationary phase. During growth at 37°C a very similar activation pattern was observed – in early exponential phase 11.2% of cells were found within EGFP-expressing gate while one generation later, in mid-exponential phase, 21.6% cells were recognised as fluorescent (Fig. 4.3B). A further increase to 28.3% was observed in late exponential phase. During the stationary phase of growth at 37°C σ^B activity did not increase at the rate observed when cells were dividing and the population of fluorescent cells remained stable with values around 35%. Based on the proportion of fluorescent cells within the population, revealed using flow cytometry of wt-egfp strain, a similar σ^B activation pattern was observed both at 37°C and 4°C, with σ^B activity increasing in the early exponential phase of growth and reaching a maximum level in stationary phase.
Fig. 4.2 Growth of fusion strains at 4°C and 37°C. Strains of *L. monocytogenes* EGD-e wild-type, ΔsigB and ΔrsbV containing P_{lmo2230::egfp} fusion, were grown at (A) 4°C and at (B) 37°C in BHI. Time points when samples of cells were taken for flow cytometry analyses were marked with a dashed line. The charts show representative results of three biological replicates carried out in duplicate in each replication (C) Specific growth rates of fusion strains grown at 4°C and 37°C were calculated for exponentially growing cultures.
©
copyright
all rights reserved
Fig. 4.3 σ^B is activated in *L. monocytogenes* EGD-e wild-type in growth phase dependent manner at 4°C and 37°C (previous page). Flow cytometry analyses of WT-egfp strain were performed with BD AcurriC6 at the interval of three days (A) and 45 min (B) at 4°C and 37°C respectively and data was processed with BD CFlow® Software. The numbers in % indicate the proportion of cells within population with fluorescence above the highest autofluorescence observed for parent WT strain. Mean fluorescence intensity (MFI) values were shown for autofluorescence range and for EGFP gate separately. Each sample was performed in biological triplicate with duplicate analyses in each replication and a minimum of 100,000 events recorded for each sample.

4.4.3. An alternative route of σ^B activation exists at 4°C in the absence of RsbV
To determine whether σ^B activity observed in wt-egfp strain (Fig. 4.3) depends solely on σ^B and RsbV during growth, similar flow cytometry analyses were performed in ΔrsbV and ΔsigB backgrounds at 4°C and at 37°C at intervals of three days and 45 min, respectively (Fig. 4.2A & 2B; Supplementary material Fig. 4.S1). All σ^B activity (i.e. EGFP-based fluorescence) was abolished in a ΔsigB background at both temperatures. Surprisingly, at 4°C cells of the ΔrsbV-egfp strain emitted fluorescence above the level of autofluorescence observed for the parent ΔrsbV strain (Fig. 4.4A). In early exponential phase (day 3) 2.2% of population was recognised as EGFP-expressing while the proportion increased to 5.1% in late exponential phase (day 6). A further increase of the fluorescent population was observed in early stationary phase of growth where it reached its highest level of 19.8% (day 9) at 4°C. In late stationary phase (day 12) population of cells emitting fluorescence above autofluorescence background decreased to the level of 10.9%. In contrast to 4°C, none of the analysed populations of ΔrsbV-egfp or ΔsigB-egfp revealed significant fluorescence above the background at any stage of growth at 37°C (Supplementary material Fig. 4.S1), suggesting that all EGFP expression observed with flow cytometry in wt-egfp population at 37°C was both σ^B- and RsbV-dependent.

To evaluate the finding of RsbV-independent expression of EGFP at 4°C microscopic observations of wt-egfp, ΔsigB-egfp and ΔrsbV-egfp cells were performed. Both the wt-egfp and ΔrsbV-egfp cultures contained fluorescent cells at 4°C, whereas none of the fields captured for a ΔsigB-egfp culture had fluorescent cells (Fig. 4.4B). To rule out the EGFP expression in ΔsigB background at 4°C (non-cell–shaped fluorescent particles), proteins were extracted from 100 ml of the fusion
strains cultures grown up to stationary phase (day 12). (Fig. 4.4C). EGFP was detected using Western blotting with anti-GFP antibody in protein extracts from the wt-egfp and low but detectable levels were observed in ΔrsbV-egfp, while no evidence of EGFP in ΔsigB-egfp extracts was seen with Western blotting. These findings suggest that a low level of RsbV-independent σB activity is present at 4°C, which is not seen at 37°C, perhaps suggesting an alternative route pathway for σB activation at low temperatures. The data presented above indicate that σB-dependent transcription at 4°C can also occur in the absence of RsbV when, according to the present model, it is expected to that σB would be bound to RsbW and unavailable for transcription. One of the possible hypotheses suggested for a similar finding in B. subtilis was related to the instability of RsbW under non-optimal temperature (Holtmann et al., 2004).

To determine whether RsbW is degraded or its levels are diminished at 4°C, Western blot analyses were performed on protein extracts from stationary phase of growth with polyclonal antibodies raised in rabbits against RsbW from L. monocytogenes EGD-e strain. Similar patterns of anti-RsbW binding was observed in wt-egfp extracts from 37°C and 4°C suggesting that temperature has no effect on RsbW degradation (Supplementary material Fig. 4.S2). Furthermore, no changes in RsbW levels were recorded between wild-type and corresponding ΔsigB and ΔrsbV mutant strains grown at 4°C indicating that these deletions do not affect RsbW stability.
Fig. 4.4 Activation of $\sigma^B$-dependent gene expression occurs in the absence of RsbV at 4°C. (A) Flow cytometry analyses of $\Delta$sigB-egfp and $\Delta$rsbV-egfp strains grown at 4°C were performed with BD AcurriC6 for cells taken at the interval of three days. The numbers in % indicate the proportion of cells within population with fluorescence above the highest autofluorescence observed for parent WT strain. Mean fluorescence intensity (MFI) values were shown for autofluorescence range and for EGFP gate separately. Each sample was performed in biological triplicate with duplicate analyses in each replication and a minimum of 100,000 events recorded for each sample. (B) Microscopy of WT-egfp, $\Delta$sigB-egfp and $\Delta$rsbV-egfp grown at 4°C and analysed in mid-exponential phase (day 6) revealed activation of $\sigma^B$ in the absence of RsbV. (C) Western blotting was carried out with anti-GFP antibody (Abcam) on SDS-PAGE separated protein extracts from stationary phase cells (day 12) of WT-egfp, $\Delta$sigB-egfp and $\Delta$rsbV-egfp grown at 4°C. Each strain was analysed in biological duplicate (A & B).
4.5 DISCUSSION

4.5.1. $\sigma^B$ activity increases during exponential phase

The main aim of this study was to examine how physiological responses are coordinated in *L. monocytogenes* by the changes in the activity of an alternative sigma factor $\sigma^B$ during growth at 37°C and at 4°C. $\sigma^B$ activity was monitored by flow cytometry using cells expressing EGFP from strongly $\sigma^B$-dependent promoter ($P_{lmo2230}$). The data show that within an exponentially growing population there is an increase in the proportion of cells displaying $\sigma^B$ activity and this continues to increase during growth, reaching maximal activation in stationary phase where ~35% of cells have fluorescence levels above background levels at both 37°C and 4°C. Previous reports showed limited activity of $\sigma^B$ in *L. monocytogenes* in exponential phase and the induction of $\sigma^B$-dependent expression during entry into stationary phase at 37°C (Becker *et al.*, 1998, Abram *et al.*, 2008a). It was therefore somewhat unexpected to observe that the largest increase in the proportion of fluorescent cells in a population expressing EGFP from the $\sigma^B$-dependent promoter of *lmo2230* occurs during exponential phase, although maximal fluorescence occurs in stationary phase. Indeed this induction of $\sigma^B$ activity occurs in exponential phase at both 37°C and at 4°C. The mechanism triggering a reprogramming of the $\sigma^B$-dependent gene expression in exponential phase is unknown at present but could be related to increasing bacterial cell density. As the cells increase in number during growth the cells experience changes in the medium composition including depletion of some specific nutrients, reduced oxygen availability, medium acidification, accumulation of metabolites and altered levels of signalling molecules. One or more of these changes could contribute to the activation of $\sigma^B$ during exponential growth and further experiments will be required to identify the specific signal involved.

4.5.2. $\sigma^B$ activity is not induced by low temperature in *L. monocytogenes*

In the present study a similar pattern of $\sigma^B$ activation was observed within populations grown in cold (4°C) or optimal (37°C) temperatures. At both temperatures $\sigma^B$ activity was found to be increased during exponential phase, reaching maximal levels of activity early in stationary phase (Fig. 4.3A & B). These data suggest that $\sigma^B$ may not play a central role in cold adaptation in
*L. monocytogenes*. This conclusion is consistent with the findings of a number of proteomic and transcriptomic studies that have sought to define the \( \sigma^B \) regulon and the cold stimulon in this pathogen. Only a very limited number of genes belonging to the \( \sigma^B \) regulon (Kazmierczak *et al.*, 2003, Hain *et al.*, 2008, Toledo-Arana *et al.*, 2009) are also found to be present in the cold stimulon (Cacace *et al.*, 2010, Chan *et al.*, 2007b, Liu *et al.*, 2002). Of the 30 genes listed as being upregulated in both exponential and stationary phase during growth at 4°C compared to 37°C (Chan *et al.*, 2007b) only two (lmo1670 and lmo1937) were described before as members of \( \sigma^B \) regulon in one study (Hain *et al.*, 2008) but not mentioned elsewhere. Furthermore, in the present study \( \Delta \text{sigB-egfp} \) and \( \Delta \text{rsbV-egfp} \) strains displayed comparable growth rates to the wild-type-egfp, demonstrating that \( \sigma^B \) activity is not critical for growth of *L. monocytogenes* EGD-e at low temperature in BHI. This is a conclusion that Chan and colleagues also highlighted in an earlier study (Chan *et al.*, 2007a). Taken together these results suggest that the modulation of \( \sigma^B \) activity that occurs during growth at 4°C is primarily influenced by the growth phase rather than the growth temperature.

### 4.5.3. RsbV-independent \( \sigma^B \) activation

Although the high degree of sequence conservation between the \( \text{sigB} \) operons in *B. subtilis* and *L. monocytogenes* suggests that regulation of \( \sigma^B \) is similar in both organisms (Hecker *et al.*, 2007, Ferreira *et al.*, 2004), this assumption has not yet been rigorously tested in *L. monocytogenes*. Initial studies on the *L. monocytogenes* system suggest that there may be fundamental differences in how \( \sigma^B \) is activated in this pathogen. Firstly, environmental (physicochemical) stress and energy stress both act via RsbT in *L. monocytogenes* (Chaturongakul and Boor, 2004), whereas energy stress is sensed in an RsbT-independent manner in *B. subtilis* (Vijay *et al.*, 2000). More recently it has been shown that energy stress signals can also influence \( \sigma^B \) activity independently of both RsbT and RsbU (Shin *et al.*, 2010a).

Many features of the upstream Rsb-dependent model regulating \( \sigma^B \) activity remain unclear in *L. monocytogenes*. Chaturongakul *et al.*, (2004) reported that RsbT contributes to \( \sigma^B \) activation through RsbV during exposure to both environmental and energy stresses in *L. monocytogenes* while in *B. subtilis* two separate pathways exist (Vijay *et al.*, 2000). A more recent study with the *opuCA-lacZ* reporter suggests
that $\sigma^B$ induction after energy stress enters the network through an RsbT-independent pathway and RsbU does not modulate that response (Shin et al., 2010a). The $\sigma^B$ regulon was not shown to be induced by any of the stimuli described thus far in $\Delta rsbV$ background and both $\Delta rsbT$ and $\Delta rsbV$ showed survival reductions similar to that of the $\Delta sigB$ strain at optimal temperature (Chaturongakul and Boor, 2006). Thus, it was surprising to note that $\sigma^B$-dependent expression occurs at 4°C in $\Delta rsbV$ background when $\sigma^B$ is expected to be completely inactivated by RsbW. RsbV-independent activation of $\sigma^B$ has been reported for chill-stressed *B. subtilis* and it was also shown to be RsbU and RsbP independent (Brigulla et al., 2003). In contrast, RsbT and RsbU were shown to be required for $\sigma^B$ induction in response cold downshift from 37°C to 7°C in *L. monocytogenes* (Shin et al., 2010a). Changes in the RsbW:SigB ratio at 4°C or possible chill-induced changes in RsbW functionality might contribute to the observed RsbV-independent effects on $\sigma^B$ activity in the present study, but further experiments will be required to clarify this point.

In conclusion, the present study shows that *L. monocytogenes* cells induce $\sigma^B$ activity in early exponential phase and $\sigma^B$ is maximally active in the population entering the stationary phase of growth. Moreover, a similar pattern of $\sigma^B$ activation is observed within populations grown in the cold and at optimal temperatures suggesting that $\sigma^B$ doesn’t play a pivotal role in cold-adaptation. Finally, we demonstrate that $\sigma^B$ activation can occur independently of the anti sigma factor antagonist RsbV in chill-stressed cells, but the mechanism underpinning this effect requires further investigation.

### 4.6. Acknowledgements

We thank members of the Bacterial Stress Response Group and colleagues in Microbiology at NUI Galway for helpful discussions and comments. This work was supported by a Science Foundation Ireland Research Frontiers Programme grant (05/RFP/GEN0044) and by an Irish Research Council for Science, Engineering and Technology EMBARK studentship together with Thomas Crawford Hayes award to MU. CB and RC were supported by Science Foundation Ireland grant SRC 09/SRC/B1794 and by a Science Foundation Ireland Stokes Professorship to RC.
Fig. 4.S1. $P_{lmo2230}$ is not activated in $L.\ monocyctogenes$ EGD-e at 37°C $\Delta \text{sigB}$ and $\Delta \text{rsbV}$ mutants (previous page)

Fig. 4.S2. RsbW levels are stable at 4°C and 37°C
CHAPTER 5

SUMMARY DISCUSSION
5.1 Introduction

Early studies of $\sigma^B$ in *B. subtilis* suggested that its regulation is complex and occurs at both transcriptional and post-translational levels (Section 1.4.1). Thus, neither *sigB* transcript levels nor protein levels of $\sigma^B$ were taken as a good indicator of how active $\sigma^B$ is in a cell. Consequently, a wide range of methodological tools were developed to monitor $\sigma^B$ activity in *B. subtilis* (Section 1.4.2.1). The methods were based on the analysis of $\sigma^B$-dependent expression to resolve the complex mechanism of $\sigma^B$ regulation, and to answer a range of questions concerning the modulation of $\sigma^B$ and its role in general stress response (Section 1.4.2.2). The present study first concentrated on developing an optimal method for tracking $\sigma^B$ activity in *L. monocytogenes* (Utratna et al., 2011, Chapter 2, Utratna et al., 2012, Chapter 3). Subsequently, the evaluated methods were used to investigate selected aspects of $\sigma^B$ modulation and to follow $\sigma^B$-dependent expression in response to osmotic stress, changing growth phase and low temperatures (Utratna et al., 2011, Chapter 2, Utratna et al., 2012, Chapter 3, Utratna et al., 2012, Submitted, Chapter 4). The aspects addressed include the timing of $\sigma^B$ activation and the ability to fine-tune the response in accordance with the extent of stress encountered. The role of RsbV as a putative anti-anti sigma factor was investigated to evaluate the model of $\sigma^B$ activation in *L. monocytogenes* under the conditions tested. Moreover, $\sigma^B$ activity was analysed at the single cell level, and heterogeneity of the $\sigma^B$-dependent response was described.

5.2. Evaluation of different methodological approaches for measuring $\sigma^B$ activity in *L. monocytogenes*

In recent years, quantitative real time RT-PCR (qRT-PCR) revolutionised the ability to measure DNA and RNA concentrations in real time during amplification, and to quantify the number of specific nucleic acid fragments in an initial template (Bustin et al., 2005). In this study, transcript levels of four $\sigma^B$-dependent genes (*sigB, opuCA, lmo2085, lmo2230*) were monitored in *L. monocytogenes* wild-type and Δ*sigB* strain, to report $\sigma^B$ activity under selected stresses (Utratna et al., 2011, Chapter 2). qRT-PCR confirmed the strong $\sigma^B$-dependence of all four genes, but reported also
substantial $\sigma^B$-independent expression of $\text{sig}B$. A short life-time of RNA allowed the resolving of a time scale of $\sigma^B$ activation after a sudden imposition of osmotic stress. A similar pattern of increasing transcript levels within 15 minutes, followed by a decrease of $\sigma^B$-dependent transcription within the next 10 minutes, was reported by qRT-PCR, for all four genes selected. The induction of $\sigma^B$ within 20-30 minutes following osmotic upshock was also demonstrated via the reporter-based approach when EGFP expression from the $\sigma^B$-dependent promoter of $\text{lmo2230}$ was observed with fluorescent microscopy (Utratna et al., 2012, Chapter 3). The delay of the $\sigma^B$ activation was attributed to the time needed for protein translation and the maturation of fluorescent protein. The transient inductions of $\sigma^B$ showed by qRT-PCR could not be observed by the measuring of OpuCA levels, or by using the EGFP reporter (Utratna et al., 2011, Chapter 2, Utratna et al., 2012, Chapter 3). Both systems rely on the comparison of $\sigma^B$-dependent expression of stable proteins, which remain present within cell even when $\sigma^B$ becomes inactive. Possible resolutions of the stability limitation have already been applied in experiments at the protein level, for other organisms, via the use of short-life reporter proteins (Hackett et al., 2006, Andersen et al., 1998). To report the dynamics of $\sigma^B$-dependent gene expression a single-cell analysis with time-lapse microscopy, coupled with mathematical recalculation of promoter activities over time, was also utilised (Locke et al., 2011, Suel et al., 2006). In this study, the temporal resolution of $\sigma^B$ activation was observed exclusively by the RNA-based approach, highlighting the usefulness of qRT-PCR in reporting $\sigma^B$ modulation in $L$. monocytogenes (Utratna et al., 2011, Chapter 2).

Whilst analysis of gene expression by qRT-PCR has provided incredible insight into $\sigma^B$-driven expression in $L$. monocytogenes, the conclusions from just measuring mRNA are limited, as they can only suggest changes in respective protein products. To evaluate whether genes transcribed by $\sigma^B$ are fully expressed and functional the protein-based approaches were utilized in this study. Firstly, antibodies against OpuCA encoding a carnitine transporter expressed in a $\sigma^B$-dependent manner were developed, and optimized to report expression driven by $\sigma^B$ (Utratna et al., 2011, Chapter 2). Secondly, a translational reporter expressing EGFP from the strongly $\sigma^B$-dependent promoter of $\text{lmo2230}$ was constructed and integrated into the chromosome of $L$. monocytogenes wild-type, $\Delta\text{sig}B$ and $\Delta\text{rsbV}$ strains (Utratna et al.,
2012, Chapter 3). The levels of $\sigma^B$ itself were also monitored under selected conditions, but, as predicted by the regulatory model, were not correlating with $\sigma^B$ activity (Utratna et al., 2012, Submitted, Chapter 4).

Protein-based approaches demonstrated clearly that the activation of $\sigma^B$ is proportional to the extent of osmotic stress. OpuCA levels measured by Western blotting were shown to gradually increase in the presence of 0.3, 0.6 and 0.9 M NaCl, respectively (Utratna et al., 2011, Chapter 2). That observation was also reported with transcript levels of four $\sigma^B$-dependent genes ($\textit{sigB}$, $\textit{opuCA}$, $\textit{lmo2085}$, $\textit{lmo2230}$) (Utratna et al., 2011, Chapter 2). Similarly, analysis by flow cytometry of EGFP expressed in a $\sigma^B$-dependent manner demonstrated an increasing proportion of cells possessing active $\sigma^B$, from 28% of an untreated population at $\text{OD}_{600}=0.6$ being fluorescent, to more than 60% of cells being fluorescent when grown in the presence of 0.9 M NaCl (Utratna et al., 2012, Chapter 3). Thus, three approaches could independently show proportional activation of $\sigma^B$ under steady-state growth in the presence of increasing concentrations of osmotic stress. However, the magnitude of induction shown at the mRNA level is much higher than that reported by OpuCA levels or the EGFP reporter approach. This finding might suggest that only a proportion of $\sigma^B$-dependent transcripts is fully translated. Additionally, this observation suggests an issue of sensitivity and saturation of detection for different methods (e.g. the moderately expressed fluorescent proteins are hard to detect due to the autofluorescence of cellular components, while extremely high levels might be toxic for bacterial cells). However, a narrow range of sensitivity might also be an advantage in some cases (e.g. the basal level of $P_{\textit{lmo2230}}$ induction attributed to the $\sigma^B$-independent expression is not detectable, see Fig. 2.S1 in Section 2.2). Thus, in this study, different approaches were combined to overcome technical limitations, for the comprehensive investigation of selected aspects of $\sigma^B$–dependent gene expression.

Following the two population-based approaches (transcript levels of $\textit{opuCA}$, $\textit{lmo2085}$, $\textit{lmo2230}$ and OpuCA levels), which measured the average value of $\sigma^B$ activity for the entire population (Utratna et al., 2011, Chapter 2, Utratna et al., 2012, Submitted, Chapter 4), a single-cell approach was used to investigate $\sigma^B$ activity at a cellular level (EGFP fluorescence measured with flow cytometry and
5.3 Regulation of lmo2230 and its putative role in L. monocytogenes

Investigations of transcriptional regulation in prokaryotes have mostly concentrated on interactions between transcriptional factors and DNA (Seshasayee et al., 2006). Thus, for years, ongoing research has not taken into account small abundant RNA-based regulation of expression. A substantial part of this study is based on measuring σB activity in L. monocytogenes by tracking the expression of egfp from P_lmo2230 (Utratna et al., 2012, Chapter 3, Utratna et al., 2012, Submitted, Chapter 4). Under selected conditions, this promoter was regarded as strongly σB-dependent, due to the absence of fluorescence in ΔsigB mutants grown under the same conditions as wild-type. Similarly, decreased fluorescence of P_{lmo2230::egfp} in wild-type was interpreted directly as lower σB activity. However, this experimental set up is not able to exclude the possibility that decreased σB activity is attributed to some P_{lmo2230} specific
regulators. For example, low fluorescence observed under some conditions might be due to the presence of a $P_{\text{lmo2230}}$ repressor, which blocks the expression of that promoter in a condition-specific manner, while $\sigma^B$ activity is actually maintained at a high level. In other words, under certain conditions, $\sigma^B$ can be active in the cell but might require some repressors to be removed, or some activators to be bound somewhere else in the selected ($P_{\text{lmo2230}}$) promoter region, to act together with RNAP for transcription. However, multiple reporter fusions consisting of other $\sigma^B$-dependent promoters would be useful in identifying promoter-specific regulators, and to avoid the wrong estimation of $\sigma^B$ activity under specific experimental conditions. Transcript mapping experiments would help clarify if there are other promoters too.

Regulatory element $\text{sreA}$ that has been shown to play a role in regulation of expression of $\text{lmo2230}$ was first identified in a tiling array study as one of seven putative S-adenosylmethionine (SAM) riboswitches, designated SreA-G (for SAM riboswitch element A-G) (Toledo Arana et al., 2009). The regulatory role for $\text{lmo2230}$ has been described in gene-array analysis comparing the $\Delta\text{sreA}$ mutant strain with the wild-type strain of $\text{L. monocytogenes}$ (Loh et al., 2009). The $\text{lmo2230}$ gene was among three genes that were expressed at higher levels in $\Delta\text{sreA}$ background, suggesting a negative regulatory role for SreA in the expression of $\text{lmo2230}$. Moreover, the levels of $\text{lmo2230}$ were shown, with Northern blots and qRT-PCR, to be restored to wild-type levels after complementing $\Delta\text{sreA}$ with SreA expressed from a plasmid. Further studies by Loh et al., (2009) investigated whether SreA has an additional function by acting as a non-coding RNA in $\text{L. monocytogenes}$. A riboswitch unable to bind SAM due to double alteration in the core of the metabolite-binding domain was constructed, and named $\text{psreA}_{\text{NSB}}$ (non-SAM binding SreA). When the $\text{psreA}_{\text{NSB}}$ construct was introduced into the $\Delta\text{sreA}$ mutant strain, the expression of $\text{lmo2230}$ was restored to the level observed in the wild-type strain. Thus, the finding indicated that the regulatory effect on the expression of $\text{lmo2230}$ is due to the sequence and structure of SreA, rather than the levels of SAM. Furthermore, SreB, showing high sequence identity to SreA, was also shown to regulate expression of $\text{lmo2230}$. The introduction of SreB into $\Delta\text{sreA}$ background was also able to restore expression of $\text{lmo2230}$ to wild-type levels.
Interestingly, both SreA and SreB were shown to be negative regulators of prfA expression (Loh et al., 2009). In the double mutant ΔsreAΔsreB, a 3-fold increase of prfA levels is observed. Expression of PrfA is controlled by SreA and SreB through the binding to the 5’UTR of prfA mRNA. Moreover, a high level of PrfA was shown to activate SreA, which in turn down-regulates prfA transcription, creating a complex feedback cycle. Regulation of lmo2230 was also reported to be influenced by PrfA (Milohanic et al., 2003). The gene was listed among genes that are σB-dependent and differentially regulated in ΔprfA background, even if they are not preceded by a putative PrfA box. Interestingly, lmo2230, has been suggested to be expressed within an operon, together with lmo2231 (Glaser et al., 2001, Milohanic et al., 2003; Hain et al., 2008). However, the genome annotation shows a terminator downstream from lmo2230 and the presence of the lmo2230-lmo2231 operon was not confirmed in the more recent global transcriptomic study (Toledo Arana et al., 2009). Based on a high similarity to arsC, from B. subtilis, lmo2230 was suggested to encode a putative arsenate reductase that might be involved in the detoxification of cells, by reducing arsenate ions (H2AsO4−) to arsenite ions (AsO2−) (Bennett et al., 2001). However, there is no experimental evidence that the protein is involved in the arsenate resistance mechanism in L. monocytogenes. Moreover, our phenotypic studies showed that even high concentrations of arsenate do not affect mutant lacking σB more than corresponding wild-type (E. Starr, unpublished data). Despite the fact that factors that activate or repress PrfA can also influence Plmo2230::egfp reporter system it still can report σB activity. The finding, showing complex regulation of lmo2230 by σB, PrfA and RNA regulators, further highlights an overlap between stress response and virulence of L. monocytogenes.

The promoter of lmo2230 has been shown to be expressed in a σB-dependent manner, and it was used in this study to monitor σB activity by measuring the expression of Plmo2230::egfp fusion with flow cytometry and fluorescent microscopy (Utratna et al., 2012, Chapter 3, Utratna et al., 2011, Chapter 2). However, despite the fact that Plmo2230 has been shown to be highly inducible, the fluorescence of the reporter protein could not be detected in cells grown on plates upon blue light illumination, which is appropriate for EGFP excitation (data not shown). This technical limitation was not resolved in this study, and did not allow the observation of σB induction by observation of fluorescence within colonies of L. monocytogenes grown on plates.
For future experiments, a system that could be used for a quick genetic screen and searching for individual colonies with increased (or decreased) $\sigma^B$ activity should be developed. It could be achieved, by fusing the lmo2230 promoter to different reporter proteins (e.g. lacZ) that could easily be differentiated on plates (blue/white screening). That approach would allow the identification of a phenotype of interest in mutagenised populations of reporter strains and select for individuals displaying induced (or reduced) $\sigma^B$ activity under selected stresses, for further analysis. The plate-based approaches were previously used to identify and characterise $\sigma^B$-dependent genes in the model organism B. subtilis and other bacteria (Boylan et al., 1991, Donegan and Cheung, 2009, Igo et al., 1987).

5.4 The role of stochasticity in the heterogeneous activation of $\sigma^B$

The population of cells of L. monocytogenes expressing EGFP in a $\sigma^B$-dependent manner exhibited a certain degree of phenotypic variability, despite no genetic differences and exposure to identical environments (Utratna et al., 2012, Chapter 3, Utratna et al., 2012, Submitted, Chapter 4). Such a cellular heterogeneity is certainly not a new observation, and was first suggested in 1953 by variability in β-galactosidase levels measured in individual cells of E. coli with a phage whose replication depended on β-galactosidase activity (Benzer, 1953). Subsequent studies explained that observation by the fluctuations in concentrations of cellular components and the random nature (or stochasticity) of biochemical reactions together with the varying collision rate of individual molecules involved in gene expression (Raser and O'Shea, 2005). Thus, in a homogenous intracellular environment, the complexity of probabilistic events (conformational changes of DNA; the range of transcriptional factors binding and unbinding for recognition of promoters, initiation and termination of transcription; the mRNA copy number and stability; translation rate, etc.) yields random fluctuations to produce a ‘noise’ in gene expression.

More recently, the phenomenon of non-genetic phenotypic variability was investigated in bacteria by expressing two fluorescent proteins of different colours
(cyan and yellow) from identical promoters on the same chromosome of *E. coli* (Elowitz *et al.*, 2002). The study showed that the expression of two identically regulated fluorescent proteins can be affected by two types of noise – extrinsic (a variation in numbers of cellular components e.g. ribosomes or RNA polymerase) and intrinsic (randomness of individual events in transcription and translation). The extrinsic noise affects both copies of fluorescent proteins in the same manner, yielding similar changes in levels of both CFP and YFP observed by cells of merged colour. In contrast, the intrinsic noise affects each copy of the promoter in an independent manner, yielding uncorrelated levels of CFP and YFP observed by cells in a range of different colours, depending on the proportion of each fluorescent protein. Moreover, a time-lapse microscopy approach showed that, in *E. coli*, extrinsic noise lasts the length of the cell cycle (40 minutes), while intrinsic noise is more rapid (shorter than 10 minutes) (Rosenfeld *et al.*, 2005). To avoid additional noise in the expression of reporter protein, a chromosomal integration of pKSV7-\(P_{\text{lmo2230}}::\text{egfp}\) was carried out for reporter strains of *L. monocytogenes* used in this study (Utratna *et al.*, 2012, Chapter 3). Thus, a single copy of the reporter gene eliminated the role of the plasmid copy number in individual cells, in the expression of the reporter fusion.

Phenotypic variability of *L. monocytogenes* expressing EGFP from \(P_{\text{lmo2230}}\) in cells grown under identical conditions was demonstrated, in this study, by flow cytometry (Utratna *et al.*, 2012, Chapter 3, Utratna *et al.*, 2012, Submitted, Chapter 4). This approach was not able to follow the temporal dynamics of \(\sigma^B\)-dependent gene expression in individual cells, but was rather a steady-state snapshot of the population at a defined stage of growth. However, it is interesting that, despite the stochasticity of processes involved and fluctuations in the concentrations of the cellular molecules, reproducible trends in \(\sigma^B\)-dependent gene expression could be defined experimentally. Thus, it is clear that the cells evolved control mechanisms for maintaining the quality of processes and possess the ability to behave robustly while using processes random in nature. The role of stochasticity in the regulation of \(\sigma^B\) activity induced under energy stress was investigated in *B. subtilis* (Locke *et al.*, 2011). The study demonstrated that noise is responsible for turning on and off a sensitive phosphorylation switch in the complex regulatory pathway of \(\sigma^B\) activation. Thus, under energy stress, the \(\sigma^B\)-dependent response is fine-tuned by the frequency
of stochastic pulses (but not the amplitude and duration) in response to fluctuations in the phosphatase/kinase ratio.

Additionally, the gene encoding $\sigma^B$ in *B. subtilis* is co-transcribed with the genes encoding its regulatory proteins involved in the partner switching mechanism of activation (Boylan *et al*., 1992) (Section 1.4.1). Moreover, $\sigma^B$ is able to autoregulate its own expression, due to the $\sigma^B$ promoter upstream of the *rsbV-rsbW-sigB-rsbX* operon (Kalman *et al*., 1990). This transcriptional feedback was shown in *B. subtilis* first to amplify, and then to terminate a pulse, causing $\sigma^B$-dependent gene expression (Locke *et al*., 2011). If kinase activity is lower than phosphatase activity, the free $\sigma^B$ further increases transcription of the operon (positive feedback). However, it also leads to increased levels of kinase (RsbW). Thus, $\sigma^B$ activation is shut off once the amount of RsbW reaches the levels sufficient to overtake phosphatase activity (negative feedback), resulting in a pulse of $\sigma^B$ activity. Overall, the mixed feedback loop regulation is based on crossing the specific threshold between kinase and phosphatase activity, and it creates pulses of $\sigma^B$ activation of a similar duration and amplitude.

The role of noise and the involvement of feedback loops in the regulation of transcriptional cascades were extensively investigated in bacteria (Becskei and Serrano, 2000, Smits *et al*., 2006). Using negative feedback loops is the most common way in which cells can suppress noise and keep the concentration of cellular components within the appropriate range of fluctuations. In contrast, the positive feedback loop behaves as a switch in which noise causing low levels of gene expression can further amplify expression of the gene itself. This type of positive regulation has been described for PrfA, one of the best studied virulence regulatory factors in *L. monocytogenes*, which can be transcribed from $P_{plcA}$ in a PrfA-dependent manner (Freitag and Portnoy, 1994). $\sigma^B$ also autoregulates its own transcription in *L. monocytogenes*, from the $\sigma^B$ promoter upstream of the *rsbV-rsbW-sigB-rsbX* operon, similar to *B. subtilis* (Ferreira *et al*., 2004, Becker *et al*., 1998, Toledo-Arana *et al*., 2009, Utratna *et al*., 2011, Chapter 2). Thus, it seems likely that noise can be utilized in *L. monocytogenes* for pulsed induction of $\sigma^B$, due to positive transcriptional feedback followed by the rapid shut-off by kinase activity of RsbW, as has been reported for *B. subtilis* (Locke *et al*., 2011). However, this idea must be
evaluated experimentally in *L. monocytogenes*, as the role of noise in $\sigma^B$-dependent gene expression has not been investigated thus far in this organism.

In this study, the induction of $\sigma^B$ activity was reported to be proportional to the extent of osmotic stress in *L. monocytogenes* using RNA-, protein- and EGFP-based reporter approaches (Utratna *et al.*, 2011, Chapter 2, Utratna *et al.*, 2012, Chapter 3). However, the mechanism of that modulation has not been explained yet in *L. monocytogenes*. The frequency of the stochastic pulses inducing $\sigma^B$ activity has been shown to be variable and modulated by the intensity of stress in *B. subtilis* – the higher the concentration of stress inducer, the more often were pulses observed (Locke *et al.*, 2011). This was in contrast to the duration and amplitude of the pulses, which were shown to be stable in nature and based on the threshold between phosphatase and kinase activity. The increasing frequency of pulses was correlated with the expression of RsbQP, which are induced in a manner proportional to the extent of energy stress. Thus, higher levels of RsbQP in a cell increase the frequency of phosphatase fluctuations and its likelihood of crossing the threshold set by RsbW kinase activity. When RsbQP was replaced with an inducible, constitutively active RsbTU complex, the frequency of stochastic pulses was also correlated with the level of expression of RsbTU. The finding suggests that increased phosphatase activity, rather than any other trait specific to RsbQP, is required to induce $\sigma^B$ activity in *B. subtilis*. Interestingly, in *L. monocytogenes*, the RsbQP pathway of $\sigma^B$ induction was not identified, suggesting that the environmental pathway is efficient to sense the extent of osmotic stress reported in this study.

### 5.5. Stress sensing and signal propagation in the $\sigma^B$ regulatory pathway

Locke *et al* (2011) showed that a rapid induction of either RsbQP or RsbTU phosphatase activity allows a crossing of the threshold set by kinase activity, leading to the activation of a $\sigma^B$-dependent stress response. However, the study did not define how an initial stress is sensed by the upper $\sigma^B$ regulatory pathway. Interestingly, in the study presented here, analysis of $\sigma^B$ activation in *L. monocytogenes* at population level showed that the cells grown under identical conditions might not be able to sense the stress in an identical way (Utratna *et al.*, 2012, Chapter 3, Utratna *et al.*, 2012, Submitted, Chapter 4). Some of the *L. monocytogenes* cells grown under
moderate stress did not express the reporter gene above autofluorescence level, suggesting that $\sigma^B$ is not induced in a portion of stressed cells, while others activate $\sigma^B$ to a very high level. In theory, the initial sensing event might create another level of $\sigma^B$ activation, being sensitive to the noise of cellular processes (e.g. the copy number of sensing molecules might vary from cell to cell). However, in other bacteria, the examples of mechanisms of non-genetic diversification within clonal populations, and their role in coping with stress and the risk of extinction, were described (Veening et al., 2008, Booth, 2002). Interestingly, the EGFP-based reporter system designed and evaluated in this study would allow the sorting of subpopulations of *L. monocytogenes* varying in levels of $\sigma^B$-dependent stress response. Their further analysis would be useful to evaluate the mechanism of diversification of phenotypes at population level, not explained thus far in *L. monocytogenes*.

Previous studies showed that, in *B. subtilis*, a large multiprotein complex called stressosome is involved in sensing environmental stress and activation of $\sigma^B$ through the RsbTU pathway (Section 1.4.1.). The stressosome is built from multiple copies of the RsbT serine/threonine kinase, RsbS and RsbR and its paralogues. RsbT is able to phosphorylate the C-terminal STAS (sulfate transporter anti-anti-sigma) domain of RsbS and RsbR, leading to RsbT release and its further interactions with RsbU. Each RsbR molecule possesses an N-terminal non-heme globin domain, forming an extension from the core of the stressosome. The turret-like domain was proposed to be a stress sensory organelle used to pass the signal to the STAS domain located downstream (Marles-Wright and Lewis, 2008). The role of selected substitutions within the N-terminal domain of RsbRA in activation of $\sigma^B$ was investigated in *B. subtilis*, with a single copy transcriptional fusion (Gaidenko et al., 2011). The study concluded that the selected mutations do not affect the ability of stress sensing *in vivo*, even if it was suggested by previous biochemical approaches (Reeves and Haldenwang, 2007, Murray et al., 2005). Thus, the study by Gaidenko et al (2011) emphasized the importance of evaluation of the *in vitro*-based models, and demonstrates the possible application of the reporter fusion system used in this study for measuring $\sigma^B$ activity in *L. monocytogenes*. The RsbR protein of *L. monocytogenes* has already been used to form stressosome in *B. subtilis*, but the stressosome itself has not been shown yet in *L. monocytogenes*, and requires comprehensive study in this pathogenic organism (Martinez et al., 2010).
One of the best studied sensory mechanisms involved in the activation of $\sigma^B$ was described for detecting light signals by *B. subtilis*. The YtvA protein, one of the RsbR paralogues, contains a single N-terminal LOV domain involved in blue-light sensing, while the RsbP protein is able to sense red light, possibly due to a structural change in the PAS domain, further activating the C-terminal PP2C-type phosphatase (Avila-Perez *et al.*, 2010, Avila-Perez *et al.*, 2006). A recent study of Lmo0799 protein from *L. monocytogenes* showed a high similarity of structure and genetic organisation to YtvA photosensor from *B. subtilis* (Ondrusch and Kreft, 2011). Moreover, the study provided the first evidence of the role of Lmo0799 in $\sigma^B$ activation upon blue-light illumination. Interestingly, light sensors in *Firmicutes* that are involved in the activation of $\sigma^B$-dependent stress response were discovered due to the identification of specific domains involved in phototaxis of other organisms (reviewed by Crosson *et al.*, 2003). Similarly, energy-dependent forms of taxis that guide bacteria to environments that provide optimal energy levels are described in model prokaryotic organisms (Taylor *et al.*, 1999). However, no direct sensors of pH, water activity, oxygen levels or temperature causing $\sigma^B$ activation have been described thus far. The question of whether these stresses are directly sensed by the stressosome or indirectly through secondary effects on the cell remains to be answered for any bacterium. The presence of multiple paralogues of RsbR suggests that each may have a different sensing capacity that can be integrated through the stressosome into the $\sigma^B$ regulatory pathway. Recently, small RNAs have been shown to play a key role in the regulation of gene expression in bacteria, in response to temperature, membrane fluidity or sensing specific molecules by riboswitches (Waters and Storz, 2009). Thus, novel methodologies and further investigation of the newly discovered and growing family of transcriptional RNA regulators might improve our understanding of how bacteria use a variety of environmental and energy-related parameters for the activation of $\sigma^B$-dependent stress response.

The role of individual members of the stressosome and other regulatory proteins in signal propagation for the activation of $\sigma^B$ is poorly understood in *L. monocytogenes*. Only selected elements of the $\sigma^B$ regulatory pathway proposed, based on genetic similarity to *B. subtilis*, have been evaluated in *L. monocytogenes*, including the role of RsbU, and the effect of *rsbT* and *rsbV* mutations (Chaturongakul and Boor, 2004, 2005).
Chaturongakul and Boor, 2006, Shin et al., 2010a). This study demonstrated the involvement of RsbV in the regulation of $\sigma^B$ activity, via the analysis of the expression of $P_{lmo2230}$::egfp fusion in the $\Delta rsbV$ background (Utratna et al., 2012, Chapter 3, Utratna et al., 2012, Submitted, Chapter 4). In accordance with the proposed model, the RsbV protein is an anti-anti-sigma factor, and is required to sequester RsbW for induction of the $\sigma^B$-dependent stress response (Section 1.4.1). Indeed, $\sigma^B$ activity was abolished in the absence of RsbV, in L. monocytogenes grown in BHI and BHI, supplemented with NaCl at 37°C. However, significant RsbV-independent activation of $\sigma^B$ was reported by flow cytometry and microscopy in sub-populations of L. monocytogenes $\Delta rsbV$ mutant grown at 4°C. Possible mechanisms of $\sigma^B$ induction in the absence of RsbV are discussed in Chapter 4. Moreover, once the $P_{lmo2230}$::egfp reporter system is well-established, it can be introduced into mutants of other regulatory proteins, to investigate their influence on $\sigma^B$ activity in future studies of stress sensing and signal propagation.

5.6. Summary and future directions

In summary, this study demonstrated that an integrated analysis of multiple levels of gene expression is essential to unravelling the different aspects of $\sigma^B$ modulation. The analysis based on RNA, which is unstable and prone to degradation, by its nature, was suited to the unravelling transient induction of $\sigma^B$ (Utratna et al., 2011, Chapter 2). The proportionate activation of $\sigma^B$ in response to the extent of osmotic stress during steady growth was reported by all three of methodologies, from increasing transcript levels of $\sigma^B$-dependent genes, increasing OpuCA levels, to proportional increases in the number of cells expressing EGFP reported when individual cells of clonal populations were analysed (Utratna et al., 2011, Chapter 2, Utratna et al., 2012, Chapter 3). The heterogeneous activation of $\sigma^B$ in clonal population was first observed via microscopy of cells expressing EGFP in a non-uniform manner (Utratna et al., 2012, Chapter 3), and was comprehensively analysed by flow cytometry under a range of osmotic stresses and during growth at optimal and low temperatures (Utratna et al., 2012, Chapter 3, Utratna et al., 2012, Submitted, Chapter 4).
The aspects of $\sigma^B$ modulation described in the present study, include its activation being heterogeneous, transient and proportional to the extent of stress. The findings provide valuable insights into understanding of regulation of this $\sigma$ factor in *L. monocytogenes*. However, the mechanisms causing the reported observations in this organism are still not well understood. Thus, further studies aiming at explaining how the stress is initially sensed from environment and searching for proteins and domains involved in this outermost element of the $\sigma^B$ regulatory pathway are highly recommended. Ultimately, these questions might be addressed by researchers that are experts in this field. However, the understanding of the behaviour of *L. monocytogenes* and mechanisms of its survival and response to stress, including antibacterial treatment, is also crucial for minimising the risk of listeriosis. A challenge for future studies will be to identify sensitive targets in order to develop efficient strategies for a complete elimination of the pathogen during food processing and storage. Elements of the $\sigma^B$ regulatory pathway might also provide sites for inhibition of $\sigma^B$ activity to limit growth, survival and virulence capacity of *L. monocytogenes*. Thus, this study, in the long term perspective, provides significant advances in that direction and may contribute to designing new therapeutic strategies and applications for food preservation.
CHAPTER 6

REFERENCES


COTTER, P. D., RYAN, S., GAHAN, C. G. & HILL, C. 2005b. Presence of GadD1 glutamate decarboxylase in selected *Listeria monocytogenes* strains is


GARNER, M. R., JAMES, K. E., CALLAHAN, M. C., WIEDMANN, M. & BOOR, K. J. 2006a. Exposure to salt and organic acids increases the ability of *Listeria monocytogenes* to invade Caco-2 cells but decreases its ability to survive gastric stress. *Appl Environ Microbiol*, 72, 5384-95.

GARNER, M. R., NJAA, B. L., WIEDMANN, M. & BOOR, K. J. 2006b. Sigma B contributes to *Listeria monocytogenes* gastrointestinal infection but not to systemic spread in the guinea pig infection model. *Infect Immun*, 74, 876-86.


MARTINEZ, L., REEVES, A. & HALDENWANG, W. 2010. Stressosomes formed in \textit{Bacillus subtilis} from the RsbR protein of \textit{Listeria monocytogenes} allow sigma(B) activation following exposure to either physical or nutritional stress. \textit{J Bacteriol}, 192, 6279-86.


OLIVER, H. F., ORSI, R. H., PONNALA, L., KEICH, U., WANG, W., SUN, Q., CARTINHOUR, S. W., FILIATRAULT, M. J., WIEDMANN, M. & BOOR,


PALMER, M. E., WIEDMANN, M. & BOOR, K. J. 2009. sigma(B) and sigma(L) contribute to Listeria monocytogenes 10403S response to the antimicrobial peptides SdpC and nisin. Foodborne Pathog Dis, 6, 1057-65.


CHAPTER 7

APPENDICES
Appendix 1
Purification of OpuCA (by Fusion Antibodies) for chicken immunisation and the development of anti-OpuCA polyclonal antibodies

Levels of OpuCA were proposed, and were later demonstrated to be a useful indication of σ^B activity in L. monocytogenes EGD-e (Utratna et al., 2011, Chapter 2). To develop a pool of polyclonal antibodies for further analysis of σ^B activity at protein level, the OpuCA protein was commercially expressed and purified by Fusion Antibodies (Belfast). The opuCA gene (lmo1428 sequence EMBL accession AL591979.1) was first cloned into pRSET, yielding pRSET-opuCA (A). Small-scale cultures of three clones with pRSET-opuCA were induced at various OD_{600}. The total protein contents were analysed by SDS-PAGE and Western Blotting, using an anti-His tag antibody (B). Clones A and B gave good expression rates were used in further experiments. 500 ml cultures were grown for each clone (A and B), and OpuCA was expressed in the conditions that were set up in the scouting. The lysates of clones A and B were purified by IMAC (HiTrap chelating 5 ml column charged with nickel). OpuCA was refolded on the column. Elution was performed using an imidazole gradient. The fractions corresponding to the elution peaks were analysed by SDS-PAGE stained with Comassie (C). The purest concentrated fractions containing OpuCA were pooled and dialysed against 20mM Tris, pH 8.5. Bradford assay was used to determine the protein concentration (D). SDS-PAGE and Western Blotting with an anti-His-tag antibody were performed to evaluate the purity and specificity of OpuCA (E). The purity of the final sample was 95%, and a 1.5 ml concentration in the final concentration of 1.2 mg ml^{-1} was obtained. The purified OpuCA was later used for the immunisation of chickens and the generation of polyclonal antibodies, as described in Fig. 2.S2.
Appx. 1. Subsequent steps illustrating purification of OpuCA by Fusion Antibodies (Belfast)

(A) Schematic illustration of the pRSET-opuCA vector (Invitrogen).
(B) Upper panel: SDS-PAGE of total protein contents from scouting. Protein extracts from three clones induced at various points of growth were analysed: non-induced culture (1), early induction $\text{OD}_{400}=0.4$ (2), normal induction $\text{OD}_{400}=0.6$ (3), late induction $\text{OD}_{400}=0.8$ (4), late induction and overnight expression (5), Benchmark Protein Ladder; (M).
Lower panel: Western blotting analysis of total protein contents from scouting with a mouse anti-His-tag antibody coupled to HRP.
(C) SDS-PAGE analysis (right panel) of the fractions collected during the OpuCA IMAC purification.
(D) Protein concentration determined by Bradford assay.
(E) Western blot (left panel) and corresponding SDS-PAGE of the final pooled fraction after dialysis.
The figure was provided by Fusion Antibodies (Belfast)
Appendix 2

The impact of other GI tract-related stresses on the expression of OpuCA

To determine whether OpuCA expression was influenced by stimuli other than osmotic stress, the expression of OpuCA under pH stress was determined. OpuCA was not detected in the ΔsigB and ΔopuCA mutants grown over a range of pH values from 7.2 to 5.0 (A). OpuCA levels in the wild-type were observed to increase with lowering pH in the exponential phase, with the highest level of expression detected at pH 5.0. Stable and high levels of OpuCA were detected in the stationary phase of growth over the whole pH range investigated. These data suggest that σ^B-dependent gene expression is influenced by pH in the exponential phase of growth, but not in the stationary phase.

The impact of other GI tract-related stresses on the expression of OpuCA was also investigated. Low levels of bile salts (up to 4.0 mM sodium cholate: sodium deoxycholate 1:1) were found to have no significant impact on OpuCA expression in the exponential phase (B). In contrast, there was a marked increase in OpuCA expression when cells were grown under oxygen-limiting conditions (C), which would prevail in the GI tract. No OpuCA was detected in the ΔsigB mutant under these conditions, suggesting that the increase seen in the wild-type was σ^B-dependent. Taken together, these data indicate that three different environmental parameters, oxygen availability, salt and pH, all influence the σ^B-dependent expression of the OpuC transporter in the exponential phase.
Appx. 2. Levels of OpuCA under conditions simulating GI environment

(A) Levels of OpuCA over a range of pH. The OpuCA protein was detected in three biological replicates of crude cell extracts, by Western blotting, using anti-OpuCA-His polyclonal antibodies, as described in Fig. 2.S2 (supplementary material). Crude cell extracts were prepared from exponential-phase cells (Exp) or stationary-phase cells (Stat) of the wild-type (wt) or the ΔsigB and ΔopuCA mutant strains grown at 37°C in BHI broth over a range of pH levels (starting pH 7.4; 6.0; 5.5 or 5.0). Total protein concentrations were normalised to 5 mg ml⁻¹, and 10 μl of each protein extract was loaded. Western blotting was carried out using semi-dry transfer, with incubations and washing steps followed by a chemiluminescent light detection, all as described in Fig. 2.S2 (supplementary material).

(B) OpuCA was detected by Western blotting in protein extracts from exponential-phase (OD₆₀₀=0.6) cells of wild-type EGD (wt), ΔsigB and ΔopuCA strains grown in BHI in the range of bile salts concentrations. Protein samples were normalised as described above.

(C) OpuCA was detected by Western blotting in protein extracts from exponential-phase cells of wild-type EGD (wt), ΔsigB and ΔopuCA strains grown in BHI and under conditions of restricted oxygen. 25 ml of BHI was inoculated to OD₆₀₀ of 0.05 in tightly closed plastic universals (25 ml), and these were incubated statically at 37°C. Cultures were inoculated with cells to give a starting OD₆₀₀ of 0.05 in 25 ml of BHI broth in 250 ml conical flasks incubated at 37°C with orbital shaking (160 rpm).
Appendix 3

Stability of the OpuCA protein

The finding that OpuCA expression was dependent on the presence of σB in every case tested suggested that OpuCA levels might act as a reliable indicator of conditions that activate σB. However, the stability of the OpuCA protein could influence its usefulness as a reporter of σB activity: if OpuCA is stable, then it would not be possible to detect a significant reduction in σB activity by measuring OpuCA levels. To determine the stability of OpuCA protein, extracts were prepared at 15-minute intervals after treatment with the protein synthesis inhibitor chloramphenicol (10 µg ml⁻¹), from both exponential-phase and stationary-phase cultures. Over a 90-minute period, there was no detectable decrease in the levels of OpuCA from either growth phase, indicating that the OpuCA protein is stable. This result suggests that, while increased OpuCA levels might indicate σB activation, it would not be possible to detect a reduction in σB activity over a short time period (90 min or less). Therefore, subsequent analyses focused on measuring σB-dependent gene expression at the mRNA level and developing a reporter system for tracking σB activity.

Appx. 3. Levels of OpuCA after stopping protein translation

The OpuCA protein was detected in three biological replicates of crude cell extracts, by Western blotting, using anti-OpuCA-His polyclonal antibodies, as described in Fig. 2.S2 (supplementary material). Crude cell extracts were prepared from cells grown up to exponential-phase cells OD₆₀₀=0.6 when chloramfenicol was added (10 µg ml⁻¹). Protein extractions were carried out at 15 min intervals. Total protein concentrations were normalised to 5 mg ml⁻¹, and 10 µl of each protein extract was loaded for SDS-PAGE. Western blotting was carried out all as described in Fig. 2.S2. Chemiluminescence was captured using Alpha Innotech FluorChem™ Imager and Fluorchem software.
Appendix 4
The activation of $\sigma^B$ is proportional to the magnitude of the pH stress at RNA level

To investigate whether a proportional activation of $\sigma^B$ in response to an increasing extent of stress is characteristic for osmotic stress only, $\sigma^B$ activity was investigated in cells grown within a pH range of 5.0 to 7.4. Relative transcript levels of three $\sigma^B$-dependent genes ($opuCA$, $lmo2230$, $lmo2085$), and two genes presumed to be $\sigma^B$-independent in the wild-type (open columns), and $\Delta$sigB mutant (closed columns) were investigated. The strains were grown in BHI broth at 37°C to the exponential phase (OD$_{600}$ of 0.6), in a range of pH concentrations (starting from pH 7.4, 6.5, 6.0, 5.5 or 5.0). RNA extracts and cDNA were prepared as previously described (Karatzas et al., 2010). All transcript levels were first normalised to the corresponding 16S RNA levels (internal control) reference gene (Tasara and Stephan, 2007), with an efficiency correction included for each primer pair, as previously described (Pfaffl, 2001), and then expressed as a percentage of the maximal level of each transcript detected in the experiment. The values presented on the graphs are the means of three independent experiments, and error bars indicate the standard deviations (n=3). Numbers shown above the graphs indicate statistically significant differences in relative gene expression (fold-change) between the stressed (pH 6.5, 6.0, 5.5 or 5.0) and non-stressed (pH 7.4) conditions ($P<0.05$ in Student’s $t$ test).
Appx. 4. Transcript levels of selected genes investigated in cells grown in the range of pH. Numbers indicate statistically significant fold changes between non-stressed and stressed conditions based on Student’s $t$ test while error bars indicate standard deviation of three biological replicates.
Appendix 5

Kinetic analysis of gene expression after weak acid upshock

Activation of $\sigma^B$ has been shown to be rapid and transient after the sudden addition of NaCl into cultures of *L. monocytogenes* EGD-e grown in BHI, up to the exponential phase (Utratna *et al.*, 2011, Chapter 2). To investigate the kinetics of *sigB* expression and three other $\sigma^B$-dependent genes (*opuCA, lmo2230, lmo2085*), together with *thiT*, presumed to be $\sigma^B$-independent, relative transcript levels were measured in exponential-phase cells in wild-type grown in DM (pH 6.40), at 37°C, shaking at 180 rpm. 20 ml cultures of *L. monocytogenes* EGD-e grown from starting OD$_{600}=0.05$ were exposed to citric (100 mM) and sorbic (10 mM) acid shock once OD$_{600}=0.6$ was reached. RNA extracts were prepared either immediately before the addition of weak acid (0 min=T0) or 10 minutes (T10), 20 minutes (T20), 40 minutes (T40) or 60 minutes (T60) after weak acid upshock. Real-time determination of gene transcription levels was carried out, as previously described (Karatzas *et al.*, 2010). All transcript levels were first normalised to the corresponding 16S RNA levels (internal control) reference gene (Tasara and Stephan, 2007), with an efficiency correction included for each primer pair, as previously described (Pfaffl, 2001), and then expressed as a percentage of the maximal level of transcript detected for each gene. One cDNA sample was generated from each of the two biological replicates, and the values presented on the graphs are the means of three independent experiments, and error bars indicate the standard deviations. Numbers shown above the graphs indicate statistically significant differences in relative gene expression (fold-change) for the wild-type between the stressed (+ weak acid at each time point) and non-stressed (time point zero before adding weak acid) conditions ($P<0.05$ in Student’s *t* test).
Appx. 5 Transcript levels of selected genes in response to weak acid shock. Numbers indicate statistically significant fold changes between non-stressed and stressed conditions based on Student’s t test.
Appendix 6
Development of the pKSV7-P_{lmo2230::egfp} reporter vector

The gene fusion construct designated P_{lmo2230::egfp} was synthesised by MWG Operon and supplied in the pBluescriptII vector. To construct an expression vector which could serve as a tool for monitoring activity of σ^B in \textit{L. monocytogenes}, all available literature was searched for i) a strong σ^B-dependent promoter and ii) an easily detectable reporter protein. A fusion was designed to include 443 bp upstream from \textit{lmo2230} (containing the region where σ^B-dependent promoter was identified) and enhanced green fluorescent protein flanked with BamHI and EcoRI restriction sites. The fusion was designated P_{lmo2230::egfp} and synthesised by Eurofins MWG Operon (A). The P_{lmo2230::egfp} construct was delivered in vector pBluescriptII yielding pBluescriptII-P_{lmo2230::egfp} (B) and transformed into \textit{E. coli} TOP10. The fusion was planned to be cloned, using BamHI/EcoRI restriction sites from pBluescriptII-P_{lmo2230::egfp}, into a shuttle vector pKSV7 (C) to generate pKSV7-P_{lmo2230::egfp} (D). However, few attempts of ligation did not result in the successful cloning of the insert into the vector. The PCR-based approach was undertaken to increase the insert:vector ratio during the ligation step. The insert was amplified with high fidelity Polymerase (Agilent) and M13 primers, then digested with BamHI/EcoRI restriction enzymes and purified with a commercial kit to remove short fragments of DNA and primers. For more information, see Section 3.3.3 and Section 3.3.4.
GAATTCC

GAATTCC

1. pBluescriptII-P<sub>lm02230</sub>::egfp cut with EcoRI and BamHI
2. P<sub>lm02230</sub>::egfp insert – PCR product with M13 primers on pBluescriptII-P<sub>lm02230</sub>::egfp
3. pKSV7 cut with EcoRI and BamHI
4. HyperLadderI
5. Empty line
6. pKSV7-P<sub>lm02230</sub>::egfp cut with EcoRI and BamHI
7. pKSV7 cut with EcoRI and BamHI
**Appx. 6. Schematic illustration of cloning steps yielding the pKSV7-\(P_{\text{lm}o\text{2}230}::\text{egfp}\) reporter vector**

(A) The sequence of the \(P_{\text{lm}o\text{2}230}::\text{egfp}\) insert synthesised by MWG Operon.
(B) The \(P_{\text{lm}o\text{2}230}::\text{egfp}\) insert delivered in pBluescriptII vector.
(C) A shuttle vector pKSV7 used for cloning.
(D) The \(P_{\text{lm}o\text{2}230}::\text{egfp}\) insert cloned into a shuttle vector pKSV7.
(E) Gel electrophoresis, and digestion of the vectors using EcoRI and BamHI, were used to confirm the correct DNA elements at each step of cloning. The presence of the \(P_{\text{lm}o\text{2}230}::\text{egfp}\) insert was confirmed within the suicide shuttle vector pKSV7.
Appendix 7
Transformation of the pKSV7-P_{lmo2230}::egfp reporter vector into \textit{L. monocytogenes} and chromosomal integration

Once pKSV7-P_{lmo2230}::egfp was developed and purified from \textit{E. coli} Top10 strain, it was introduced to \textit{L. monocytogenes} EGD-e wild-type, ΔsigB and ΔrsbV strains by electrotransformation. The resulting transformant strains had pKSV7-P_{lmo2230}::egfp that could be confirmed using PCR on colony templates with M13 primers resulting in a 1200 bp product. When the transformants were incubated at 42°C the vector was not able to replicate, and was forced to integrate by homologous recombination within the P_{lmo2230} region of the chromosome. The integration could be confirmed with PCR on colony templates with COB688/689 primers, resulting in a 660 bp product. Agarose gel electrophoresis of PCR products was used to follow various genetic manipulations used to construct the egfp fusion strains in \textit{L. monocytogenes} EGD-e background.

Appx. 7. Electrotransformation of \textit{L. monocytogenes} EGD-e wild-type, ΔsigB and ΔrsbV strains with pKSV7-P_{lmo2230}::egfp, and the development of fusion strains
Letters A and B represent two colony templates of wild-type strain; letters C represent colony templates of ΔsigB, while letters D represent colony templates of ΔrsbV strain. Products of the amplification with M13 primers on strains with pKSV7-P_{lmo2230::egfp} integrated into chromosomes are shown in lanes 1 to 4, while lanes 12 to 15 represent the amplification of corresponding strains transformed with the vector, but before integration was carried out. Lanes 8 to 11 illustrate the products of amplification, with COB688/689 primers on integrants. PCR with M13 on purified pKSV7-P_{lmo2230::egfp} (positive control) is shown in lane 5, while PCR on ddH2O templates (negative controls) for M13 and COB688/689 are shown in lanes 7 and 8, respectively. HyperLadderI was run in lane 16 for the purpose of size determination.
Appendix 8
Optimisation of quantification fluorescence microscopy

Once pKSV7-P$_{lmo2230}$-egfp was constructed, it was transformed into L. monocytogenes and shown to be expressing a functional EGFP. Then reliable methodology for measurements of fluorescence exhibited by cells expressing EGFP, and effective image processing techniques were required to extract and quantify relevant information. The quantitative fluorescent microscopy involved appropriate (i) sample preparation, (ii) stringent image acquisition and (iii) image processing methods. All the steps of sample preparation were carried out in the presence of a maximally reduced amount of light in the preparation room, using non-transparent light protective tubes to minimise fluorescence bleaching. A public domain program, ImageJ (developed at the US National Institutes of Health and available on the internet at http://rsb.info.nih.gov/ij), was selected as the most suitable package for the processing and analysis of the microscopic images obtained. Thus, strict and consistent sample preparation techniques, image acquisition devices and the corresponding computerised image analysis were applied in this study for the preserving and quantification of σ$_B$-driven fluorescence. For further details concerning the steps involved in quantitative fluorescent microscopy, see Appendices 9-10.

Appx. 8. Optimisation of fluorescence microscopy for quantification with ImageJ. Elements of optimisation are shown in boxes while subsequent steps are indicated as bullet points.
Appendix 9
Fixation and filtration of L. monocytogenes fusion strains for quantitative microscopy

In order to stop the physiological processes of bacterial cells and protect fluorescence signals at specific points during growth, cells of L. monocytogenes EGD-e wild-type, ΔsigB and ΔrsbV integrants were treated by ice-cold methanol/ethanol fixation (A). This fixation methodology was developed by a series of control experiments, in order to establish a treatment that preserved cellular morphology, and to avoid permeabilisation. In the final protocol used throughout this study, OD<sub>600</sub> of cultures was measured, and a cell suspension in BHI equivalent to 1 ml of OD<sub>600</sub>=0.6 was prepared. Then, the cell suspension was immediately mixed with 1 volume of ice-cold 1:1 methanol/ethanol (v/v) mixture, briefly vortexed and kept for 10 minutes at -20°C. Subsequently, the samples were centrifuged at 9,000 g for 10 minutes at 4°C, to pellet the fixed cells and remove alcohol/media residues. After centrifugation, the supernatant was gently discarded and the cells were resuspended in 1 ml of sterile PBS of pH 7.4. Samples were either stored at 4°C for immediate analysing, or frozen at -20°C in 1 ml aliquots for later processing.

To obtain reliable images for fluorescent quantification of bacteria, a flat surface covered with dispersed and non-overlapping cells was needed. Thus the bacterial cells were filtered onto a filter (Millipore) with 0.2 µm pore, 10 mm in diameter. The filtration unit (Millipore) was assembled to contain the filter (Millipore), which was placed between two paper pads (Whatman). Before the filtration, the bacterial cells were fixed and resuspended in 1 ml of sterile PBS of pH 7.4, as described above. 1 ml of this suspension was mixed with 9 ml of PBS and placed into the funnel. A vacuum suction was applied to allow a concentration of suspended bacterial cells on the surface of the filter. The filter was removed carefully with tweezers and transferred to a microscope slide containing 3 µl of mineral oil (Sigma-Aldrich) spread as a mounting medium. 3 µl of mineral oil was also placed and spread in the middle of a cover slip (18 mm x 18 mm), which was then put upside down on the top of the filter. Finally, the cover slip was pressed carefully onto the slide to dispense the mounting oil equally over the filter. The same mineral oil was used with a 100x magnification lens in further microscopic analysis. The amount of cells taken for the
filtration step may need to be varied (more or less than 1 ml of OD\textsubscript{600}) if significantly different levels of EGFP expression are observed under different experimental conditions. The sample preparation, filtration and filter mounting were standard to all treatments (A). Before analyses amoles were thawed on ice in a dark container for min 1 hour and fluorescence signal was confirmed to be stable when tested with flow cytometry.

<table>
<thead>
<tr>
<th>A</th>
<th>Start of bacterial growth</th>
<th>Bacterial culture (BC) OD\textsubscript{600}=0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sampling point OD\textsubscript{600} measurement</td>
<td>OD\textsubscript{600} &lt; 0.6</td>
<td>OD\textsubscript{600} = 0.6</td>
</tr>
<tr>
<td>Calculations for fixing step</td>
<td>Ex. OD\textsubscript{600} = 0.4 BC [ml] x 0.4 = 1 [ml] 0.6 BC=1.5 [ml]</td>
<td>BC=1 [ml]</td>
</tr>
<tr>
<td>Dilution of BC</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Fixing</td>
<td>ice cold methanol:ethanol 1:1 (v/v) 1.5 ml +BC = 1.5 ml</td>
<td>ice cold methanol:ethanol 1:1 (v/v) 1 ml +BC = 1 ml</td>
</tr>
<tr>
<td>Brief vortexting</td>
<td>Incubation 10 min at -20 °C</td>
<td></td>
</tr>
<tr>
<td>Resuspension of fixed cells</td>
<td>Centrifugation 10 min at 4 °C 9,000 g</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Removal of supernatant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Resuspension of cells in 1 ml of PBS pH 7.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fixed bacterial cells OD\textsubscript{600}=0.6 in PBS</td>
<td></td>
</tr>
</tbody>
</table>
Appx. 9. A schematic illustration of fixation (A) and filtration (B) procedures for quantification of fluorescence with microscopy.
Appendix 10
Background subtraction and pixel intensity measurement of digital images, with ImageJ

For each experimental condition, a number (>30) of microscopic fields was captured for further image analysis. Firstly, to correct for unevenly illuminated backgrounds and background noise, a command, “Process > Background Subtraction”, using a “rolling ball” algorithm developed by Stanley Sternberg (Sternberg S, Biomedical image processing, IEEE Computer, Jan 1983), was applied to all images (A). Here, a region of interest (ROI) was selected to demonstrate the result of subtraction algorithm on pixel intensity profiles via “Analyse > Plot profile” (B). An effect of the background subtraction on the 3D profile of the pixel intensities within the whole image processed was shown by the “Analyse > Surface Plot” command (C). Furthermore, the numerical outcome following background subtraction was compared for untreated and treated files, by the grey-scale histogram. Each pixel is placed in a bin corresponding to the colour intensity of that pixel, added up and displayed on a graph. A histogram of the image represents the distribution of the pixels over the grey-level scale (0-255; black-grey-white). In later analyses, the mean pixel intensity of individual images with subtracted background was quantified with ImageJ, and the resulting data was exported to Microsoft Excel. The relative fluorescence intensities between selected conditions (e.g. stressed and untreated) were determined by assigning a value of 1 to the lower mean intensity recorded among selected experimental conditions. Student’s t test was used to determine the statistical significance of ratio differences between groups.
Appx. 10. Comparison of intensity measurements between two microscopic images with: non-subtracted background (left) and subtracted background (right). Original files (A) were first analysed by profiling 2D of selected ROI (B) followed by 3D profile (C). Then fluorescence intensity levels were compared between non-subtracted and subtracted pictures.
Appendix 11

Particle counting with ImageJ

It has been observed that cells bearing egfp expressed in a σ^B-dependent manner not only differ in their fluorescence intensities, but that some are not fluorescent enough to be detected by selected microscopy settings despite being exposed to identical conditions. Thus, another approach that was utilised to compare σ^B activity was based on the calculations of the number of cells exhibiting fluorescence within an optimised number of cells filtered and captured by fluorescence microscopy. For automatic particle analysis, an image with a previously subtracted background is first opened in ImageJ (A), and a simple segmentation technique (thresholding algorithm) is applied to distinguish the object from the background using the menu command “Image>Adjust>Threshold” (B). A threshold limits pixels in the image to black (background), while pixels with values outside the defined range are converted to white (objects) (C). To reduce user-dependent bias of manual thresholding, algorithms automatically calculating the threshold are recommended (Sezgin and Sankur, 2004). Once the threshold is defined, a binary image (black/white) is created using the menu command “Process>Binary>Make binary” (D). Due to the possible presence of slightly overlapping elements, objects in a binary image are then separated by a watershedding algorithm, using the menu command “Process>Binary>Watershed” (E). Then, objects can be analysed in a segmented image, using the menu command “Analyze > Analyze particles” (F). Additionally, the minimal size of objects included in particle counting can be defined; this was set to 5x5 (25 pixels) throughout this study, to minimise the influence of the background noise (G). Finally, a summary table is displayed by the software (H), and the data can be exported for statistical analysis to a Microsoft Excel spreadsheet, using the menu command “File>Save as>.xls file”. Moreover, a sequence of commands can be recorded via Macro using the menu command “Plugins>Macros>Record”, to avoid multiple manipulations and perform all the tasks in an automatic manner.
Appx. 11. Subsequent commands used for particle counting within images obtained with fluorescence microscopy. An original file is opened in Image J (A) and thresholding is applied (B) to distinguish between objects and background (C). The image is converted into a binary file (D) and overlapping elements are separated by watershedding (E). Then particles are automatically counted (F) after setting the limit of the size recognised as a cell (G). Results are summarised within a table (H).
Appendix 12

Calculation of the proportion of fluorescent cells with fluorescence microscopy

Once the protocol for particle counting with ImageJ was developed, it could be used for the comparison of particles captured by phase contrast and fluorescence microscopy within the same field (A). The quantification would allow an assessment of the proportion of cells (% of fluorescent population) that expressed \textit{egfp} under selected conditions, and were captured by a previously described set of manipulations (sample preparation, filter, exposure time, etc). The phase contrast image is first inverted using the menu command “Edit>Invert” (B). Then, the protocol of particle counting (Appendix 11) is applied for two images of the same microscopic field (C and D). The number of particles counted in each image is displayed in a summary table, and is used for defining the proportion of cells expressing \textit{egfp}. Additionally, a manual count can be performed by either the “Point Picker” (E) or “Cell counter” plugin, for the exclusion of particles that are clearly not objects of interest, arising from non-adjustable equipment limitations.
A
RAW
TIF File

B
Invert
TIF file

C
Adjust
Threshold
Appx. 12. Calculation of the proportion of cells expressing *egfp* within a population captured by phase contrast and fluorescence microscopy with ImageJ.
Appendix 13
Optimisation of flow cytometry and defining the gate for $\sigma^B$-dependent expression of egfp

To define the proportion of cells being fluorescent within a population of WT-egfp fusion strain, microscopy was first utilised (Appendix 12). However, the microscopy-based approach requires the filtration of a fixed cell suspension, the capturing of multiple fields in both phase contrast and fluorescence mode, followed by image analysis. In contrast, flow cytometry allows automated, and less laborious, determination of the fluorescence level of each cell within a fixed cell suspension. Additionally, by analysis of the fluorescence of $\Delta\text{sigB-egfp}$ grown under similar conditions, a range of fluorescence levels attributed to $\sigma^B$-dependent expression of P$_{lmo2230}$ could be defined within the WT-egfp population. Flow cytometry was carried out in the laser-based BD Accuri C6 flow cytometer (Accuri Cytometers, Inc.) with the assistance of BD CFlow software. To define a range of $\sigma^B$-dependent fluorescence (gate) cells of WT-egfp (positive) and $\Delta\text{sigB-egfp}$ (negative) strains grown in the presence of 0.5 M NaCl and fixed at OD$_{600}=0.6$, then, they were run through 488 nm blue laser (excitation). The data detected in the FL1 533 / 30 nm (e.g., FITC/GFP) emission channel were analysed for 100,000 of events (particles) for each strain. BD CFlow software allowed a plotting of the fluorescence level (SSC) versus the relative size of the particle (FSC). The plot was used for defining a gate of fluorescence levels (inside the red box) with values above autofluorescence level (fluorescence attributed to $\sigma^B$ activity). Next, the number of events detected (Count) for each fluorescence level was represented as a histogram, with the gate threshold applied automatically by BD CFlow software, in a vertical line. Thus, the proportion of cells being fluorescent due to $\sigma^B$-dependent expression of egfp could be defined automatically by software as a numerical value expressed as a percentage of the fluorescent population.
**Appx. 13. Initial optimisations of FCM settings**

100,000 events for both WT-egfp (positive) and ΔsigB-egfp (negative) strains grown in the presence of 0.5 M NaCl and fixed at OD_{600}=0.6 were tested with BD Accuri C6 flow cytometer.

(A) The plot showing fluorescence (SSC) versus the relative size of the particles (FSC) was used for defining a gate of autofluorescence levels.

(B) Histograms were used to illustrate abundance of each fluorescence level within tested population. Numbers in red indicate percentage of population exhibiting fluorescence below (left) and above (right) autofluorescence threshold (vertical line).
Appendix 14

Activation of σB in L. monocytogenes grown in the range of salt stress investigated at a single cell level

Once the gate of fluorescence exhibited by cells due to σB-dependent expression of egfp was defined (Appendix 13), analysis of WT-egfp and ΔsigB-egfp strains grown in the presence of 0 M, 0.3 M, 0.6 or 0.9 M NaCl, fixed at OD₆₀₀=0.4, was performed. Additionally, corresponding non-fusion strains (WT and ΔsigB) were grown and analysed under similar conditions, to evaluate the gate defined before. Interestingly, both non-fusion strains were characterised with autofluorescence levels described before for theΔsigB-egfp fusion strain (data shown for WT strain only). Thus, the results suggest that the presence of the integrated construct does not affect the autofluorescence exhibited by cells under the conditions investigated. Moreover, an increase in the proportion of the population being fluorescent at OD₆₀₀=0.4 was shown to correlate with the extent of stress applied. However, a smaller percentage of the population induced σB activity when compared to the population of cells investigated with the same methodology at a later point of growth, at OD=0.6 (see Fig. 3.6).
Appx. 14. Heterogeneity of fluorescence within an EGFP-expressing population, and σ^B activation proportional to the extent of osmotic stress at OD=0.4. The same data is presented as dot plots (A) and histograms (B) for comparison. The vertical lines indicate threshold for autofluorescence.
Appendix 15

Activation of \( \sigma^B \) at the single cell level after treatment with antimicrobials

To investigate whether sub-inhibitory concentrations of antibacterial agents used in medical and industrial treatments against \( L. \ monocyctogenes \) can rapidly activate \( \sigma^B \), upshock experiments were performed at \( \text{OD}_{600} = 0.2 \), with disinfectants and compounds active against both the cellular membrane and peptidoglycan. Firstly, growth experiments were conducted to determine the concentrations for each of the compounds that inhibit SGRs of \( \text{wt-egfp} \) strain by \( \sim 25\% \) - benzalkonium chloride (BC) (0.5μg ml\(^{-1}\)), ampicillin (0.0625μg ml\(^{-1}\)), nisin (2.0 μg ml\(^{-1}\)), gramicidin (3.3 μg ml\(^{-1}\)) and hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) (0.025% v/v). These concentrations were used to stimulate a stress response without causing cell death in experiments carried out for \( \text{wt-egfp}, \Delta \text{sigB-egfp} \) and \( \Delta \text{rsbV-egfp} \), together with their parent non-fusion strains (used to determine autofluorescence levels). In upshock experiments, rotary-shaken (180 rpm) cultures were grown at 37°C from \( \text{OD}_{600} = 0.05 \) to \( \text{OD}_{600} = 0.2 \), which corresponded to the early exponential phase of growth when the sample was first taken (0 minutes) and fixed for FCM. Cultures were subsequently treated with sub-inhibitory concentrations (25% reduction of \( \text{wt-egfp} \) SGR) of antibacterial agents, and sampling was repeated 30 min and 60 min after the treatments, from vigorously shaken cultures incubated at 37°C. A 30% (v/v) stock of \( \text{H}_2\text{O}_2 \) and the remaining chemicals in powder form were obtained from Sigma-Aldrich. Aqueous stocks of BC (100 mg ml\(^{-1}\)), ampicillin (5 mg ml\(^{-1}\)) and nisin (1 mg ml\(^{-1}\)) were used, while gramicidin stock (20 mg ml\(^{-1}\)) was prepared in ethanol. Control experiments for the equivalent volume of ethanol alone were performed to confirm that diluents had no effect alone.

Based on the flow cytometry data, a statistically significant (\( p < 0.05 \)) induction of \( \sigma^B \) could be detected within 60 min of treatment with a sub-inhibitory concentration of BC, when 33.1% of the population was recognised as fluorescent in comparison to 28.2% of the untreated population. The sub-inhibitory concentration of \( \text{H}_2\text{O}_2 \) increased \( \sigma^B \) activity above the level observed in the untreated population, also within 60 min, when 45.1% of population became fluorescent. Both in the case of ampicillin-treated and gramicidin-treated cells, a shift in the proportion of EGFP-expressing cells within the population was not significantly above the increase
reported for untreated cultures; thus, no effect by ampicillin and gramicidin can be observed within 60 minutes of treatment. No effect on the fluorescence of population treated with ampicillin and gramicidin was surprising, as they cause peptidoglycan and membrane-related stresses, respectively. However, the result could possibly be affected by the short timing of the experiment, limited to 60 minutes after the treatment, or may be explained by the low concentrations of inhibitors used to avoid cell lyses and the leakage of the cytoplasmic content into the extracellular environment. After the addition of nisin (2.0 μg ml⁻¹), commonly used as a food preservative (E234), a significant (p < 0.05) increase of σB-dependent EGFP expression occurred within 30 minutes: 35.4% of the population was fluorescent, compared to 22.4% of the untreated population at the same time. The proportion of EGFP-expressing nisin-treated cells was stable over the following 30 minutes, and still significantly above the level of the untreated population (34.6% and 28.2%, respectively). This could possibly be explained by its dual mode of antibacterial action – nisin affects both the cell wall and membrane permeability by sequestering the main transporter of peptidoglycan subunits (lipid II) and using it to form pores through the membrane (reviewed by Cotter et al, 2005). In contrast, the ΔrsbV-egfp and ΔsigB-egfp strains were not fluorescent above autofluorescence levels after any of the treatments mentioned above. In summary, nisin treatment can rapidly activate σB in the early exponential phase of growth, and the addition of both BC and H₂O₂ induce σB within 60 minutes of treatment in an RsbV-dependent manner. Based on the data presented in this study, low working concentrations of antilisterial compounds can paradoxically induce a rapid σB-dependent stress response and enhance resistance to compounds used for disinfecting and food preservation. In conclusion, the results obtained in this work provide a new insight into the understanding of the physiological mechanisms behind the survival of cells within a population, and might help in the designing of new methods and planning appropriate dosages that inactivate *L. monocytogenes* populations in complex food-processing environments, without inducing its stress response.
Appx. 15. An investigation of $\sigma^B$ activity in *Listeria monocytogenes* EGD-e after treatment with selected antimicrobial compounds: (A) benzalkonium chloride and hydrogen peroxide; (B) Ampicillin, Gramicidin and Nisin. Histograms represent population of *L. monocytogenes* EGD fixed before (first column), 30 min) after (middle column) and 60 min after (right column) treatments with (A) benzalkonium chloride and hydrogen peroxide (B) ampicillin, gramicidin and nisin. Numbers represent percentage of fluorescent populations and stars show statistically significant differences based on Student’s $t$ test analysis ($P<0.05$).
Appendix 16

Development of fusion strains in *L. monocytogenes* 10403S wild-type, ΔsigB and ΔrsbV background

pKSV7-P_<sub>lmo2230</sub>::egfp was introduced to *L. monocytogenes* 10403S wild-type, ΔsigB and ΔrsbV strains by electrotransformation. Then integrants were developed in *L. monocytogenes* 10403S background, as described in Appendix 7 for *L. monocytogenes* EGD-e. To follow various genetic manipulations used to construct the egfp fusion strains, agarose gel electrophoresis of PCR products was used (A). Bacterial strains and plasmids used for developing fusion strains in the *L. monocytogenes* 10403S background are listed (B).
Appx. 16. Development of egfp fusion strains in *L. monocytogenes* 10403S background

(A) Products of the amplification with M13 primers on colony templates of *L. monocytogenes* 10403S strains transformed with pKSV7-Plmo2230::egfp are shown in lanes 2 to 4. Lanes 6 to 8 and 9 to 11 represent amplification with COB688/689 and M13 primers, respectively, on colony templates from corresponding strains, with the vectors integrated into chromosomes. HyperLadderI was run in lines 1, 5 and 12, for the purpose of size determination. (B) A list of plasmids and strains used in the development of egfp fusion strains in *L. monocytogenes* 10403S background.
Copyright
All rights reserved
©  
copyright  
all rights reserved
©  copyright  all rights reserved
©
copyright
all rights reserved
©
copyright
all rights reserved