Molecular Investigation into the Roles of Sigma B and the Stressosome in the Response of *Listeria monocytogenes* to Environmental Stress

By

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Abstract

The alternative sigma factor, SigB, has been studied extensively in *Listeria monocytogenes, Bacillus subtilis* and *Staphylococcus aureus,* and is known to regulate the general stress response in these bacteria. The activation of SigB by a stress occurs via a signalling cascade, upstream of which is the stressosome complex. The stressosome is a large protein complex composed of RsbS, RsbT and RsbR proteins, with the RsbR proteins hypothesised to function as stress sensors. RsbR has four paralogues, Lmo0161, Lm0799, Lmo1642 and Lmo1842, of which only Lmo0799 has been established to act as a stress sensor. Lmo0799 is a blue light sensor, but somewhat surprisingly inactivation of the corresponding gene does not result in sensitivity to blue light.

In the current study, environmental factors including growth phase and temperature, along with exposure to ethanol or alternative carbon sources, were identified to alter the sensitivity of *L. monocytogenes* to killing by blue light. Whole transcriptomic analysis of the wild-type, Δ*sigB* mutant and a mutant for which the conserved cysteine residue critical for blue light sensing in Lmo0799, C56, has been changed to an alanine, designated *lmo0799* C56A, in the presence of blue light identified a key role for SigB in altering gene transcription in response to blue light exposure. In addition, it confirmed the ability of the C56A mutant to upregulate genes under the control of SigB in response to blue light exposure, although to a lesser extent than the wild-type. These data provide further evidence that *L. monocytogenes* is able to sense and respond to secondary stresses associated with blue light exposure, such as ROS. Finally, the reconstruction of Δ*lmo1642* and Δ*lmo1842* deletion mutants enabled the confirmation that there is a redundancy between the stress sensing abilities of these proteins

and the other RsbR paralogues that has previously been associated with Lmo0161.

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List of Abbreviations

µg	Microgram
µl	Microlitre
ALA	Aminolevulinic Acid
APS	Ammonium persulphate
ATR	Acid tolerance response
BHI	Brain Heart Infusion
BLUF	Blue-Light-utilising flavin adenine dinucleotide
bp	Base pairs
cDNA	Complimentary DNA
CFU	Colony forming units
dH_2O	Deionised water
DM	Chemical defined medium
DNA	Deoxyribonucleic Acid
eGFP	Enhanced Green Fluorescence Protein
g	Gram
GABA	γ-aminobutyrate
GAD	Glutamate decarboxylase
kDa	Kilo-Dalton
LB	Luria-Bertani
LLO	Listeriolysin O
LOV	Light-Oxygen-Voltage
M	Molar
mA	Milli-amp
mg	Milligram
ml	Millilitre
mM	Millimolar
mRNA	Messenger RNA
ms	Millisecond
mW	Milliwatt

NADPH	Nicotinamide adenine dinucleotide phosphate
NB	Nutrient broth
nm	Nanometer
OD	Optical Density
PAS	Per-Arnt-Sim
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PTS	Phosphotransferase System
PVDF	Polyvinylidene Difluoride
PYP	Photoactive Yellow Protein
RAST	Rapid Annotations using Subsystems Technology
RNA	Ribonucleic Acid
RNA seq	RNA Sequencing Analysis
ROS	Reactive Oxygen Species
RPM	Revolutions per minute
rRNA	Ribosomal RNA
RT-PCR	Real-Time Polymerase Chain Reaction
SAM	S-adenosylmethionine
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate - Polyacrylamide gel electrophoresis
SGR	Specific Growth Rate
SGWB	Sucrose Glycerol Wash Buffer
SNP	Single Nucleotide Polymorphism
sRNA	Small RNA
ssDNA	Single Strand DNA
STAS	Sulphate Transporter and Anti-Sigma Factor
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween
TEMED	Tetramethylethylenediamine
WGS	Whole Genome Sequencing

Chapter 1: Introduction

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1.1 Classification

The genus *Listeria* is composed of Gram positive bacilli with a low G+C content (Vazquez-Boland *et al.* 2001). Initially, only six species were characterised: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. grayi,* with only *L. monocytogenes* and *L. ivanovii* being pathogenic (Volokhov *et al.* 2002), but this has been expanded to 17 species over recent years: *L. marthii*, *L. fleischmannii*, *L. floridensis*, *L. aquatica*, *L. newyorkensis*, *L. cornellensis*, *L. rocourtiae*, *L. weihenstephanensis*, *L. grandensis*, *L. riparia*, and *L. booriae* (Orsi and Wiedmann 2016). *L. monocytogenes* was first described in 1926 by Murray *et al.* when it was isolated in a blood culture from a pregnant rabbit (Murray *et al.* 1926). It was not isolated from a human infection until 1929, when it was isolated by Neyfeldt (Farber and Peterkin 1991).

L. monocytogenes has been divided into four lineages, lineages I, II, III and IV, based on the results of studies investigating the phylogeny and subtyping of the species (Orsi *et al.* 2010). Of the four lineages, the majority of *L. monocytogenes* isolates can be categorised into either lineage I or II (Orsi *et al.* 2010). In order to further characterise isolates, *L. monocytogenes* can be divided into 13 serotypes based on somatic (O) and flagellar (H) antigens: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, 7 (Nadon *et al.* 2001, Jaradat and Bhunia 2003). For the purpose of epidemiological studies, serotyping is of limited usefulness, owing to approximately 95% of isolates from human infections belonging to serotypes 1/2a, 1/2b and 4b (Ryser and Marth 2007).

1.2 *L. monocytogenes* in the environment

L. monocytogenes is a ubiquitous organism in the environment but can cause infection when it enters the food chain. Common sources of *L. monocytogenes* in the food chain include ready-to-eat food products e.g. deli meats and smoked salmon, unpasteurised milk and dairy products e.g. soft cheeses, and raw sprouts (US Department of Health and Human Services 2018). *L. monocytogenes'* presence in the environment is partly due to its high level of tolerance to environmental stresses. In addition, the bacterium can utilise a range of carbon sources, including glucose, glycerol, fructose, mannose (Tsai and Hodgson 2003), cellobiose, trelose (Abram *et al.* 2008) and lactose (Dalet *et al.* 2003), which increases the variety of habitats it can colonise.

In order to metabolise this variety of carbon sources, *L. monocytogenes* has developed a varied range of sugar transport (Deutscher *et al.*, 2014). A high number of ATP-binding cassette (ABC) transporters and PTS systems have been identified in the genome, though the majority of tthese have not yet been characterised. The role of glucose and glycerol in virulence and stress gene regulation have been well studied (Raengpradub *et al.* 2008, Deutscher *et al.* 2014). It has been suggested that glucose metabolism is inhibitive of PrfA activity, the main regulator of virulence in *L. monocytogenes* (Deutscher *et al.* 2014), while glycerol metabolism activates PrfA, thereby increasing virulence (Joseph *et al.* 2008). A role for the general stress response regulator Sigma B in glycerol metabolism has also been defined (Abram *et al.*, 2008). Taken together, these studies indicate that the metabolism of particular carbon sources may influence the transcription and regulation of genes in *L. monocytogenes,* potentially acting as an indicator to the organism as to whether it is inhabiting the environment or the host.

1.3 Disease and pathogenesis

L. monocytogenes predominantly infects immunosuppressed individuals, including pregnant women, neonates and the elderly, although it can infect any individuals to cause a disease known as listeriosis (Liu 2006). Listeriosis can present in many ways including meningitis, bacteraemia, encephalitis, endocarditis and febrile gastroenteritis, with certain presentations occurring more frequently in some groups than others (Schlech and Acheson 2000). Upon the development of an infection, the global mortality rate of listeriosis is approximately 20-30% (World Health Organisation 2018), reduced in the United States to approximately 16% (Centers for Disease Control and Prevention 2017). Due to its common persistence in food, it is likely that healthy individuals build immunity to *L. monocytogenes* by maintaining a population of memory T cells targeted against the bacterium (Vazquez-Boland *et al.* 2001).

After ingestion, the bacteria are transported to the gastrointestinal tract. In most individuals, *L. monocytogenes* will be unable to cause an infection, and the bacteria will be excreted to the environment in faeces. In some individuals, *L. monocytogenes* may cross the intestinal epithelial barrier and translocate to the liver or spleen via the bloodstream and lymphatic system (Cossart and Toledo-Arana 2008). After replicating in hepatocytes, large numbers of cells can reach the blood, brain and placenta, leading to often fatal infections (Cossart and Toledo-Arana 2008).

The incidence of listeriosis in neonates can be classified as either early-onset or late-onset neonatal listeriosis. Early-onset neonatal listeriosis arises when bacteria cross the placental barrier and the mother delivers an infected infant, whereas late-onset listeriosis is caused by infection of the

infant either during or immediately after delivery (Schlech and Acheson 2000). The symptoms differ slightly between the two classifications of neonatal listeriosis. Early-onset neonates are likely to suffer from acute sepsis, but other symptoms including respiratory distress and pneumonia, meningitis and endocarditis, while late-onset neonates present most commonly with meningitis and septicaemia (Baxter *et al.* 2009). In cases where early-onset neonatal listeriosis is suspected, histological examination of the placenta for lesions and the presence of Gram positive bacilli can aid diagnosis (Teixeira *et al.* 2011). A 10 year study into neonatal listeriosis in the UK confirmed 19 cases, with 18 being confirmed as early-onset neonatal listeriosis, for which the mortality rate was 21% (Sapuan *et al.* 2017), demonstrating the high mortality rate for this usually rare disease.

In *L. monocytogenes,* SigB controls the general stress response and virulence is controlled by Positive Regulatory Factor A (PrfA). PrfA has two upstream promoters (*prfAp*$_1$ and *prfAp*$_2$), with *prfAp*$_2$ being SigB-dependent (Nadon *et al.* 2002). A whole transcriptome comparison of gene expression levels in the intestine compared to Brain Heart Infusion broth identified altered expression levels for 1206 genes, of which 232 were regulated in a SigB-dependent manner (Toledo-Arana *et al.* 2009). When the same analysis was carried out in blood, a similar number of genes showed altered expression, however many genes with a SigB promoter and a PrfA binding site were altered in a PrfA-dependent but SigB-independent manner (Toledo-Arana *et al.* 2009). This alteration in transcriptional regulation indicates a complex overlap between PrfA and SigB in virulence gene expression and suggests a switch between these regulators as the infection progresses beyond the gastrointestinal tract.

1.3.1 Role of SigB in virulence, independently of PrfA

Upon ingestion, *L. monocytogenes* is first challenged by the acidic conditions of the stomach, where it encounters pH values between 2.0 and 2.5. An investigation into the role of SigB in the activation of virulence genes, found that a Δ*sigB* mutant showed reduced transcription of *inlA, lmo0669, opuCA* and *bsh* compared to the wild-type under non-stress conditions, and also that the Δ*sigB* mutant was unable to significantly increase the transcription of these genes upon exposure to acid or salt stress (Sue *et al.* 2004).

From the stomach, *L. monocytogenes* is transported to the gastrointestinal tract where the processes of adhesion and internalisation begin (Mengaud *et al.* 1996, Vazquez-Boland *et al.* 2001). InlA and InlB are two of the internalin proteins produced by *L. monocytogenes,* under the control of SigB (Kazmierczak *et al.* 2003), enabling the cells to bind to human E-cadherin and Hepatocyte Growth Factor Receptor (HGFR) proteins (Bonazzi *et al.* 2009). By binding to E-cadherin, InlA induces rearrangements of the cell cytoskeleton which is critical for internalisation of *L. monocytogenes* into the host epithelial cell (Hamon *et al.* 2006). Likewise, the binding of InlB to HGFR also induces cytoskeletal rearrangements but, in addition, facilitates clatherin-mediated endocytosis (Bierne *et al.* 2007). Invasion of both epithelial and hepatocyte human cell lines is significantly reduced in a mutant lacking σ^B, and this correlates with a reduction in *inlAB* transcription in this strain (Kim *et al.* 2005).

The OpuC operon of *L. monocytogenes* is involved in the uptake of carnitine and glycine betaine in response to osmotic stress (Sleator *et al.* 2001). In both *L. monocytogenes* ScottA and LO28 strains, the inactivation of the *opuC* operon results in reduced colonisation of the small intestine in the mouse virulence model (Sleator *et al.* 2001). In addition to *opuC, L. monocytogenes* also encodes two additional osmolyte transporters in its genome, *betL* and

gbu, however their deletion from the genome does not significantly alter virulence of the organism (Wemekamp-Kamphuis *et al.* 2004). Unlike the other transporters, OpuC is the only transporter able to transport carnitine, a molecule that is readily available in mammalian cells, suggesting that carnitine is required for *L. monocytogenes* virulence (Wemekamp-Kamphuis *et al.* 2004). However, *ΔopuC* mutant is able to utilise carnitine as an osmoprotectant when there are high levels of extracellular carnitine, suggesting that a secondary carnitine transport system, with a low affinity for carnitine, exists (Fraser and O'Byrne 2002). In the gastrointestinal tract, bile stress is one of the stresses experienced by the pathogen, with a role for SigB in bile tolerance clearly defined (Zhang *et al.* 2011, Gahan and Hill 2014). Further studies into the requirement of OpuC for virulence identified a bile-sensitive phenotype for the Δ*opuC* mutant, a phenotype which could be reversed through the addition of exogenous carnitine (Watson *et al.* 2009).

1.3.2 The role of PrfA in virulence

PrfA is the primary controller of virulence genes within *L. monocytogenes,* with the Δ*prfA* mutant being avirulent (Chakraborty *et al.* 1992). The main virulence genes under the control of PrfA include *prfA, hly, plcA, plcB, mpl* and *actA* (Fig. 1.1) (Scortti *et al.* 2007). Due to the SigB-dependent PrfA promoter, *prfAp*$_2$, the transcription of *prfA* is partially SigB -dependent, with the Δ*sigB* mutant showing reduced virulence in the murine infection model (Nadon *et al.* 2002).

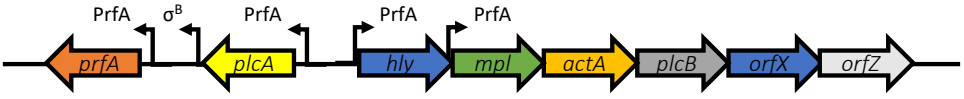

Figure 1. 1 The PrfA operon of *L. monocytogenes.*
Each gene of the PrfA operon is represented by an open arrowhead. Transcription can be initiated from the promoters represented by angled arrowheads, which are under the control of either PrfA or SigB.

Based on its structural and functional similarity to the *Escherichia coli* cyclic AMP receptor protein, PrfA can be grouped into the group of transcriptional regulators known as the Crp/Fnr family (Kreft and Vázquez-Boland 2001). Unlike other regulators in the Crp/Fnr family, PrfA does not contain the conserved glycine residues required to bind to cAMP, however it does contain the conserved regions required to bind to RNA polymerase (Kreft and Vázquez-Boland 2001). In its active or dimerised state, PrfA is able to bind to a designated PrfA box (Dickneite *et al.* 1998) located -41.5 bp upstream from the ribosome binding site (Vazquez-Boland *et al.* 2001, Scortti *et al.* 2007). Due to a PrfA promoter located upstream of *prfA,* this regulator is subject to autoregulation (Scortti *et al.* 2007)

From the intestine, *L. monocytogenes* adheres to the surface of host epithelial cells, resulting in the formation of a vacuole (Vazquez-Boland *et al.* 2001). The binding of the bacterium to the epithelial cell wall is mediated by InlA and InlB which are under the control of SigB (Hamon *et al.* 2006, Bierne *et al.* 2007). The escape of the bacterium from this vacuole is dependent upon listeriolysin O (LLO), a secreted pore-forming toxin encoded by *hly* (Gedde *et al.* 2000). Upon escape from the vacuole, expression of *actA* enables the recruitment of the Arp2/3 complex and polymerisation of host cell actin to generate an actin tail, enabling the bacteria to propel themselves through the host cell membrane and into neighbouring cells (Toledo-Arana *et al.* 2009). Upon entry into the neighbouring cells, the bacteria are enclosed in secondary vacuoles, which LLO and PlcA/B are once again required in order to escape (Carvalho *et al.* 2014).

The transcriptional study by Toledo-Arana *et al.* (2009) showed a strong downregulation of genes composing the virulence locus (*prfA-plcA-hly-mpl-actA-plcB-orfX-orfZ*) in the intestine, and strong upregulation of the same genes in the blood; however, the roles of LLO and ActA in escaping the host cell vacuole and recruiting and polymerising host cell actin, respectively,

suggest that the activation of PrfA occurs before the bacteria enter the bloodstream. A study investigating the role of LLO in cell-to-cell spread suggests that LLO is required only for the escape from the host cell vacuole, and not for the formation of the vacuole (Gedde *et al.* 2000, Lebreton *et al.* 2015).

1.3.3 The link between SigB and PrfA in virulence

A study into the regulation of genes by PrfA identified a set of genes whose expression is also regulated by SigB, indicating that the PrfA and SigB regulons may overlap (Milohanic *et al.* 2003). At present, the mechanism of interlink between SigB and PrfA is unknown. In the intestine, 232 genes under the control of SigB show significant changes in transcription including *inlA* and *inlB* (Toledo-Arana *et al.* 2009). Upon crossing the intestinal membrane into the blood, expression of virulence genes, including *inlA* and *inlB*, increases in a PrfA-dependent SigB-independent mechanism (Toledo-Arana *et al.* 2009). In *L. monocytogenes* 10403S, 10 genes have been identified that are co-regulated by SigB and PrfA (Chaturongakul *et al.* 2011).

The control of virulence gene expression at 30°C is independent of PrfA. At lower temperatures, the Shine-Dalgarno site of *prfA* is masked by a secondary RNA structure, inhibiting *prfA* expression at temperatures below that of the host (Johansson *et al.* 2002). The expression of *prfA* is thought to be further regulated by *sreA*, an S-adenosylmethionine (SAM) riboswitch, in low-nutrient environments (Loh *et al.* 2009). In low-nutrient environments such as the intestine, expression of *sreA* is increased and the levels of the ncRNA increase, enabling it to bind to the 5'-UTR of *prfA* and inhibit translation (Loh *et al.* 2009). Together, these complex regulatory mechanisms seem to prevent the expression of *prfA* at lower temperatures and in low-nutrient environments, conditions where the activity of SigB is

increased. Kazmierczak *et al.* (2006) hypothesised that the activation of SigB by exposure to environmental stresses primes the bacteria for intracellular infection, by increasing expression of *prfA* via the SigB-dependent *prfA*p_2 promoter prior to the bacteria becoming intracellular. The additional regulation of *prfA* translation via the RNA thermometer and SAM riboswitch prevents translation of PrfA prior to intracellular infection (Johansson *et al.* 2002, Loh *et al.* 2009).

1.3.4 Galleria mellonella as a model for *L. monocytogenes* infection

The suitability of the *G. mellonella* infection model for *L. monocytogenes* was first assessed in 2010 (Joyce and Gahan 2010, Mukherjee *et al.* 2010). *G. mellonella* represent a good infection model for *L. monocytogenes* as it can be incubated at 37°C, and no ethics approval is required (Mukherjee *et al.* 2010). The inoculation of *G. mellonella* with a selection of strains indicated that it was a suitable model of infection for differentiating between highly virulent and less virulent strains (Mukherjee *et al.* 2010). Moreover, the mortality rate was linked to the initial pathogen load (Mukherjee *et al.* 2010). Taken together, these results suggest that *G. mellonella* larvae represent a good model of infection for *L. monocytogenes* that is representative of human infection.

When the virulence of a series of mutants was examined, a requirement for *hly* was shown at both 30°C and 37°C, and a requirement for *prfA* at 30°C only (Joyce and Gahan 2010). In addition, mutants lacking *virR,* involved in regulating *L. monocytogenes* surface components, *actA* or *plcA* showed a significant reduction in virulence compared to the wild-type (Rakic Martinez *et al.* 2017). These studies confirmed that the previously identified virulence regulon under the control of PrfA (Chakraborty *et al.* 1992) was also required for virulence in *G. mellonella.* Further work identified a role for *actA* and *hly*

in the infection of *G. mellonella* with *L. monocytogenes* in the formation of actin tails, as is the case in human infection (Mukherjee *et al.* 2013).

More recent work studying the suitability of *G. mellonella* as an infection model for *L. monocytogenes* has demonstrated its suitability for investigating anti-*Listerial* compounds and routes of infection and immunity (Mukherjee *et al.* 2013). Furthermore, studies investigating the potential of probiotics to reduce the severity of infection by *L. monocytogenes* have been conducted in *G. mellonella,* showing that probiotics may be useful in reducing the severity of infection by *L. monocytogenes* (Grounta *et al.* 2016).

1.4 The general stress response

The bacterial general stress response involves the transcription of a large number of genes under the control of a regulatory factor in response to the detection of environmental stress (Hecker and Volker 2001). In *B. subtilis* and *L. monocytogenes,* the general stress response is under the control of the alternative sigma factor Sigma B (σ^B) (Becker *et al.* 1998, Hecker and Volker 1998), while in *E. coli* the general stress response is under the control of RpoS (σ^S) (Lange and Hengge-Aronis 1991). In the unstressed cell, gene transcription is under the control of a housekeeping sigma factor, however the detection of an environmental stress signal leads to the binding of the general stress response sigma factor to the catalytic core of RNA polymerase (Feklístov *et al.* 2014). A promoter sequence upstream from a gene is complimentary to a specific sigma factor, enabling the sigma factor to bind to the DNA strand and guide the RNA polymerase to begin transcription of the gene (Feklístov *et al.*, 2006). Therefore, the general stress response genes have a promoter sequence which is complementary to the general stress response sigma factor, allowing strict regulation of their transcription. It should be noted that genes can have more than one promoter sequence,

enabling them to be transcribed by a variety of sigma factors. Furthermore, a gene is not required to have a SigB promoter to be activated by SigB as a secondary transcription factor that is activated by SigB can work in concert with SigA. The SigB operon is detailed in Fig. 1.2(A).

1.4.1 The current model of SigB regulation in *Listeria*

While evidence for the existence of a stressosome complex within *L. monocytogenes* has only recently been obtained (Impens *et al.* 2017), previous work has demonstrated that replacement of the *rsbR* gene in *B. subtilis* with the *L. monocytogenes rsbR* gene, allows for activation of the SigB signalling cascade (Martinez *et al.* 2010). In addition, BLAST comparisons between the *L. monocytogenes* and *B. subtilis* genomes have shown high levels of sequence homology between components of the stressosome and SigB signalling cascade (Ferreira *et al.* 2004). Therefore, research into the stressosome complex and SigB signalling cascade in *B. subtilis* has provided a solid foundation to guide research into the same areas within *L. monocytogenes.*

In the unstressed *B. subtilis* cell, SigB is sequestered by the anti-sigma factor RsbW (Regulator of Sigma B), inhibiting its interaction with RNA polymerase, and the SigB regulon is not transcribed (Ferreira *et al.* 2001). Upstream of RsbW, the anti-anti-sigma factor, RsbV, is phosphorylated and unable to bind RsbW in its phosphorylated state (Yang *et al.* 1996). In a stressed cell, RsbU acts as a phosphatase, dephosphorylating RsbV, and enabling the binding of RsbV to RsbW (Yang *et al.* 1996). As a result, SigB is left free to bind to the core enzyme of RNA polymerase, and transcription of the SigB regulon occurs (Hecker *et al.* 2007).

The stressosome is a 1.8 megadalton protein complex that acts upstream of RsbU as a signal integration hub, enabling the activation of the SigB signalling

cascade in response to environmental stress (Fig. 1.2B) (Marles-Wright *et al.* 2008). Composed of RsbR, RsbS and RsbT protein subunits, the stressosome is thought to sense stress through the N-terminal region of RsbR, leading to the phosphorylation of RsbR and RsbS by RsbT, and subsequently the dissociation of RsbT from the stressosome (Chen *et al.* 2003). After its dissociation from the RsbS:RsbR complex, RsbT binds to RsbU, and activates the phosphatase activity of RsbU (Marles-Wright *et al.* 2008). Phosphorylated RsbV is the substrate for RsbU and the reaction results in a dephosphorylated form of RsbV that can interact directly with the anti-sigma factor RsbW, thereby liberating σ^B to associate with the transcriptional apparatus.

The continual activation of SigB is deleterious to the cell, and mutants with constitutively phosphorylated RsbS generate small colonies (Min Kang *et al.* 1996). Similarly, the deletion of SigB from the genome can confer a growth advantage in the absence of environmental stress (O'Donoghue *et al.* 2016) In the absence of stress, the phosphatase protein RsbX dephosphorylates RsbS, enabling RsbT to reassociate with the RsbR:RsbS complex instead of with RsbU (Chen *et al.* 2003). Computational modelling of RsbX phosphatase activity suggests that RsbX dephosphorylates RsbS at a higher rate than it does RsbR (Liebal *et al.* 2013).

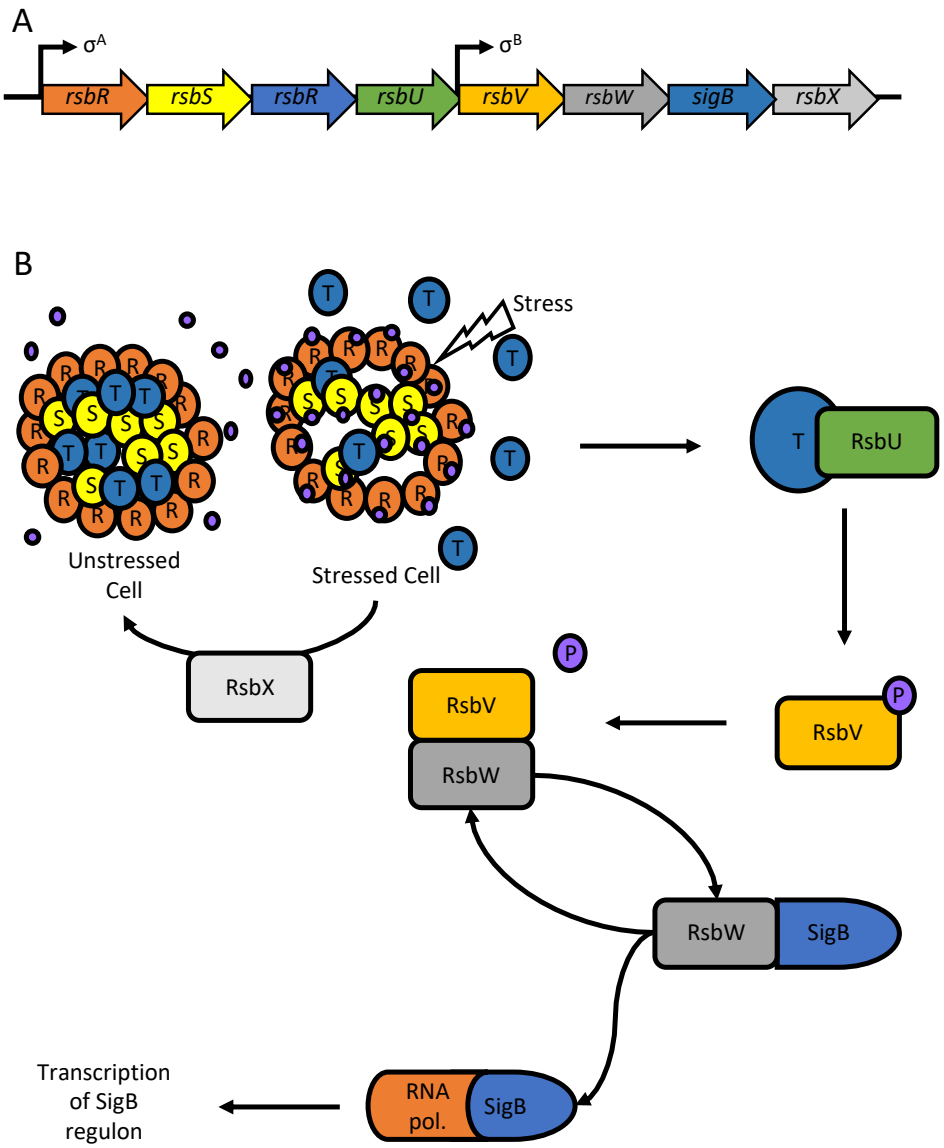

Figure 1. 2 The SigB signalling cascade.

(A) Each gene of the SigB operon is represented by an open arrowhead. Transcription can be initiated from either of the two promoters represented by angled arrowheads, and the putative terminator sequence is represented by a stem and loop structure. (B) Upon perception of a stress, RsbT dissociates from RsbR and RsbS of the stressosome complex and binds to RsbU. RsbU becomes active as a phosphatase and facilitates the dephosphorylation of RsbV. The anti-sigma factor, RsbW, is bound to SigB in an unstressed cell, but has a higher affinity for RsbV in it unphosphorylated state, leading to its dissociation from SigB in a stressed cell. Sigma B is free to bind to RNA polymerase, and initiate transcription of the SigB gene regulon.

1.4.2 RsbU

RsbU is the first protein in the SigB signalling cascade downstream from the stressosome. A BLAST search of the *rsbU* nucleotide sequence identified homologous sequences in more than 15 bacterial species, including *Bacillus spp., Listeria spp.,* and *Staphylococcus spp.* In *B. subtilis*, RsbT complexes with the N-terminal region of RsbU, mostly with the first 84 amino acids of RsbU, following its dissociation from the stressosome (Delumeau *et al.* 2004). Despite the role of RsbT in activating RsbU phosphatase activity, a Δ*rsbT* mutant, but not a Δ*rsbU* mutant, is still able to respond to starvation stress through SigB activation (Shin *et al.* 2010). While these results show an essential role for RsbU in responding to energy stress via activation of SigB, it is likely that RsbU can be activated via a mechanism independently of RsbT. In *L. monocytogenes* there is genetic evidence suggesting that RsbT is essential for the activation of σ^B (Chaturongakul and Boor, 2004).

1.4.3 RsbV

Downstream from RsbU, in the unstressed cell, the anti-anti-sigma factor RsbV exists in its phosphorylated state, and unable to bind to RsbW. In experiments investigating the role of RsbV in surviving environmental stress treatments, the Δ*rsbV* mutant exhibited to the same phenotype as the Δ*sigB* mutant when challenged with synthetic gastric fluid, acid (pH 2.5) and cumene hydrogen peroxide (Chaturongakul and Boor 2004), suggesting that RsbV is required for the activation of SigB under these conditions. In a similar study, the Δ*rsbV* mutant showed a reduced growth rate compared to the isogenic parental strain when challenged with mild osmotic, acid (pH 4.5), or alcohol stress (Zhang *et al.* 2013). The measurement of SigB activity via RT-PCR targeted against the SigB-dependent gene *opuCA,* showed reduced levels of *opuCA* transcripts in the Δ*sigB* and Δ*rsbV* mutants

compared to the wild-type after exposure to osmotic, acid or ethanol stress (Chaturongakul and Boor 2006). Taken together, these studies show a requirement for RsbV to activate SigB in response to certain environmental stresses. In the absence of RsbV, it is likely that RsbW remains bound to SigB, and therefore SigB is unable to interact with RNA polymerase and initiate transcription of the general stress response genes required to induce a protective response.

Interestingly, during growth in carbon-limited defined medium, both the Δ*rsbV* and Δ*sigB* mutants exhibit an increased growth rate compared to the wild-type, but also an increased death rate upon entry into stationary phase (Chaturongakul and Boor 2006). When the transcript levels of *opuCA* were measured after 6 h and 12 h growth in the same medium, both the Δ*sigB* and Δ*rsbV* mutants showed significantly lower levels of *opuCA* relative to the wild-type (Chaturongakul and Boor 2006). While the exact mechanism for such a result is unknown, one reason for the increased growth rate of the Δ*sigB* and Δ*rsbV* mutants in carbon-limited medium might be related to the competition that exists between the housekeeping Sigma factor, SigA, and SigB (O'Byrne and Karatzas 2008). In the presence of SigB, less RNA polymerase is available to SigA to drive the transcription of genes involved in cell growth; but in the Δ*sigB* and Δ*rsbV* mutants, this competition has been removed and so more cell resources are available to drive cell growth.

1.4.4 The structure of the stressosome

The stressosome is composed of RsbR, RsbS and RsbT proteins, with approximately 40 copies of RsbR and 20 copies each of RsbS and RsbT (Marles-Wright and Lewis 2010). The structure is composed of a core region made up of RsbS:RsbT complexes, into which the C-terminal region of RsbR is embedded, leaving the N-terminal region of the RsbR protein to protrude out into the cell (Marles-Wright and Lewis 2010). In addition to RsbR, four

proteins with high levels of sequence similarity to RsbR are thought to co-exist with RsbR in the stressosome complex, but the stoichiometry remains largely unknown. In *B. subtilis,* RsbR and its paralogs are designated RsbRA, RsbRB, RsbRC, RsbRD and YtvA, with their homologs in *L. monocytogenes* designated RsbR (*lmo0889)*, Lmo0161, Lmo0799, Lmo1642 and Lmo1842 (Ondrusch and Kreft 2011).

1.4.5 The sensory mechanisms of the stressosome

While the existence of a stressosome complex in *L. monocytogenes* has been described (Impens *et al.* 2017), the roles of RsbR and its paralogues in stress sensing remains unknown. The exception to this is the Lmo0799 protein (YtvA in *B. subtilis*), which has been characterised as a blue light sensing protein (Ondrusch and Kreft 2011). Novel work using N-terminomics has identified a previously undescribed miniprotein, Prli42, that is essential for SigB activation in response to oxidative stress (Impens *et al.* 2017). It is thought that Prli42 anchors RsbR to the bacterial membrane by interacting with the N-terminal domain of RsbR (Impens *et al.* 2017). The N-terminal domains of RsbR and its paralogues are not conserved, showing only 17-22% sequence identity compared to 45-50% sequence identity for the C-terminal regions (Murray *et al.* 2005).

Despite the high level of variability in the N-terminal structures of RsbR and its paralogues, there is a level of redundancy in their sensing function (Kim *et al.* 2004). The construction of triple knockout mutants in *Bacillus subtilis* showed that, while there was a variation in the efficacy of stress sensing between the paralogues, all of the paralogues individually were able to sense and respond to ethanol stress (Kim *et al.* 2004). More recent work has shown that cells expressing only a single paralogue of RsbR have an altered response profile to ethanol stress (Cabeen *et al.* 2017). While all mutants were able to activate SigB in response to 2% ethanol stress, the speed,

duration and magnitude of their responses differed, leading the authors to conclude that each of the paralogues contributed individually to the response (Cabeen *et al.* 2017).

1.5 The resistance of *L. monocytogenes* to environmental stress

L. monocytogenes is of particular concern to the food processing industry due to its high level of resistance to commonly utilised food preservation techniques. The role of the alternative Sigma factor SigB in coordinating the response to osmotic stress in *L. monocytogenes* was first reported by Becker *et al.* (1998). Since this, SigB has been implicated in the resistance of *L. monocytogenes* to a plethora of environmental stresses, including, but not limited to, osmotic (Fraser *et al.*, 2003; Utratna *et al.*, 2011), pH (Wemekamp-Kamphuis *et al.* 2004, Zhang *et al.* 2011), temperature (Liu *et al.* 2002) and oxidative stress (Ferreira *et al.* 2001). In addition to its role in initiating the general stress response, SigB is also implicated in the formation of biofilms by *L. monocytogenes* (van der Veen and Abee 2010).

1.5.1 Osmotic stress

Salting is a widely used preservation technique used within the food processing industry, and so overcoming osmotic stress is essential for a foodborne pathogen to survive in the food chain. *L. monocytogenes* has been shown to withstand up to 20 h exposure to 7 M NaCl, equivalent to 40% (w/v), salt concentrations (Liu *et al.* 2005). In order to survive such challenging conditions, the bacterium employs several mechanisms to overcome osmotic stress. One of these is the uptake of compatible solutes, including glycine betaine, glutamate and carnitine, from the extracellular environment (Tombras Smith, 1996; O'Byrne and Booth, 2002).

Investigations into the compatible solutes accumulated by *L. monocytogenes* in response to osmotic stress identified glycine betaine as the predominant osmolyte, increasing 20-fold in cells exposed to osmotic stress compared to the unstressed control (Ko *et al.* 1994). The primary glycine betaine uptake system is encoded by *betL* (Sleator *et al.* 1999), which has a SigB promoter -33 bases upstream (Fraser *et al.* 2000). When a Δ*betL* mutant is cultured on solid agar supplemented with 4% NaCl, the colonies exhibit a pinprick morphology compared to the isogenic parent strain (Sleator *et al.* 1999). In a growth assay comparing the ability of the wild-type and Δ*sigB* mutant strains to accumulate glycine betaine in response to osmotic stress, the wild-type strain was able to accumulate glycine betaine at approximately double the rate of the Δ*sigB* mutant, showing a partial role for SigB in glycine betaine accumulation (Fraser *et al.* 2003).

In addition to glycine betaine, carnitine is a second important compatible solute involved in osmotic stress tolerance. *L. monocytogenes* transports carnitine into the cell via the OpuC transport system, encoded by the *opuCA,CB, CC, CD* operon, which has a SigB promoter upstream of *opuCA,* with carnitine uptake being completely abolished in the Δ*opuC* mutant (Fraser *et al.* 2000). Similarly to the Δ*opuC* mutant, carnitine uptake is almost completely abolished in the Δ*sigB* mutant (Fraser *et al.* 2003). In exponentially growing cells, the level of OpuCA detected by Western blot analysis increases in proportion to the level of osmotic stress encountered by the cells, with no protein detected in the Δ*sigB* mutant (Utratna *et al.* 2011). Carnitine transport has also been shown to be important for growth and survival in the murine gastrointestinal tract (Sleator, Wouters *et al.*, 2001; Sleator and Hill, 2010).

1.5.2 Acid Stress

L. monocytogenes is exposed to a wide range of pH values in both the food processing environment, and after ingestion into the human digestive tract. Within a human, the bacterium is subjected to the highly acidic environment of the stomach (typically pH 2), and also the more alkaline environment of the duodenum (pH ~6). In order to overcome the stresses associated with this rapidly changing environment, *L. monocytogenes* possesses a wide array of mechanisms it can employ when required. These systems include, but are not limited to, the glutamate decarboxylase (GAD) system (Cotter *et al.* 2001), arginine deaminase system (Ryan *et al.* 2009), and the adaptive acid tolerance response (ATR) (Davis *et al.*, 1996; O' Driscoll *et al.* 1996), all of which, at least partially, require SigB.

The GAD system is encoded by the five *gad* genes, A-E, of which the transcription of all except for *gadA* is induced in the wild-type strain after exposure to pH 4.5 for 1 h (Wemekamp-Kamphuis *et al.* 2004). Upon exposure to acid, extracellular glutamate is transported into the cell via either of the two glutamate/ γ-aminobutyrate (GABA) antiporters, GadT1 or GadT2, and converted to GABA by the Gad enzymes, GadD1, D2 and D3 (Feehily *et al.* 2013, Gahan and Hill 2014), a decarboxylation reaction that consumes a proton and so contributes to reducing the acidity of the cell cytoplasm (Karatzas *et al.* 2010). In addition to the removal of protons from the cell cytoplasm, GABA is less acidic than glutamate so its accumulation in the cell cytoplasm also contributes to an increase in pH (Cotter *et al.* 2001). When transcription of the *gad* genes were measured in the Δ*sigB* mutant, only transcription of *gadE* was induced under the same conditions, confirming the functionality of the SigB promoters upstream from the *gadCB* operon and *gadD* (Wemekamp-Kamphuis *et al.* 2004). However, when the levels of GABA were measured in the wild-type and Δ*sigB* mutant after exposure to pH 2.5 for 1 h, there were no significant differences in the levels

of GABA detected (Ferreira *et al.* 2003). When considered together, these results suggest that while SigB is involved in regulating the GAD system, there may be an alternative mechanism by which the production of GABA is regulated.

In a similar manner to the GAD system, the ADI system increases the cytoplasmic pH through the conversion of arginine to ornithine, carbon dioxide and ammonia, with the ammonia being converted to ammonium via the addition of an intracellular proton (Ryan *et al.* 2009). The proposed model for the ADI system suggests that arginine is either transported into the cell via the ArcD transporter, or synthesised from glutamate via the arginine synthesis pathway (Gahan and Hill 2014). The conversion of arginine to citrulline is regulated by SigB via the ArcA protein; ArcA is encoded by *lmo0043* which has a SigB promoter -73 bases upstream, and its transcription is decreased in a Δ*sigB* mutant (Hain *et al.* 2008).

The ATR of *L. monocytogenes* requires *de novo* protein synthesis during exposure to mildly acidic conditions, allowing the bacterium to adapt to and survive lethal acidic conditions (Davis *et al.* 1996). While SigB is required for acid tolerance, it is not of major importance to the ATR. In the absence of both SigB and pre-exposure to mild acid conditions, the cells show almost a 1000-fold greater reduction in cell numbers over 3 h when exposed to pH 2.5, compared to the wild-type (Ferreira *et al.* 2001). When the cells are exposed to pH 4.5 for 1 h prior to exposure to pH 2.5, the wild-type and Δ*sigB* mutant strains show 10-fold and 100-fold reductions, respectively, after 3 h (Ferreira *et al.* 2001). In a similar study, the role of SigB in the ATR was assessed at different phases of growth, with a greater requirement for SigB identified as the cells approached stationary phase compared to exponential phase (Ferreira *et al.* 2003).

From the studies presented above, it is clear that SigB is involved in the response of *L. monocytogenes* to acid stress. However, the extent to which it is required is dependent upon the level of stress encountered, and the protective mechanism required for the response. The responses utilised by *L. monocytogenes* upon encountering acid stress, including the GAD system and the ATR, require a number of complex pathways, likely involving number of regulators in addition to SigB. More research will be necessary to fully elucidate the regulatory interactions that occur during acid stress and to clarify fully the role of SigB in the response.

1.5.3 Oxidative stress

Oxidative stress can be defined as 'the shift in balance between oxidant/antioxidant in the favour of oxidants', and destructive consequences arise when the antioxidant mechanisms of the cell are overcome (Birben *et al.* 2012). The three most physiologically important categories of reactive oxygen species (ROS) include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) (Imlay 2013). The negative consequences of ROS on cells include DNA damage, lipid peroxidation, and oxidative damage of proteins (Bandyopadhyay *et al.* 1999; for a detailed review see Cabiscol *et al.*, 2000).

DNA damage that results from interactions with ROS can occur in the form of base degradation, breaks within DNA strands and helices, and mutations within the genetic code (Birben *et al.* 2012). Research investigating the role of SigB in the protective response of *L. monocytogenes* to oxidative stress, found that the Δ*sigB* mutant was 100-fold more sensitive to killing by oxidative stress than the wild-type, suggesting that SigB is at least partly responsible for resistance to oxidative stress (Ferreira *et al.* 2001). Three genes identified as having a role in the resistance of *L. monocytogenes* to oxidative stress, *lmo0515, lmo1580* and *lmo2673,* were also shown to have

upstream SigB promoters (Seifart Gomes *et al.* 2011), while the recently identified miniprotein responsible for tethering RsbR to the cell membrane, Prli42, is required in order to mediate the activation of SigB by oxidative stress. Despite this evidence for the role of SigB in oxidative stress tolerance, Boura *et al.* (2016) have shown that the presence of SigB is deleterious for the cell at stationary phase in the presence of hydrogen peroxide. Likewise, the same deleterious role for SigB in hydrogen peroxide stress resistance has been shown in *Bacillus cereus* (van Schaik *et al.* 2005). This apparent paradox shows that further research will be required to clarify to involvement of SigB in the response to oxidative stress.

1.5.4 Blue light stress

While the induction of the SigB-dependent genes *lmo0596* and *lmo2230* in response to blue light requires Lmo0799 (Tiensuu *et al.* 2013), the deletion of *lmo0799* from the genome does not increase the sensitivity of *L. monocytogenes* to killing by blue light (O'Donoghue *et al.* 2016). This suggests that the bacteria can sense and respond to alternative stresses associated with photodynamic inactivation (PDI) via an alternative sensory mechanism. In addition, while SigB is required for resistance to lethal blue light, its absence is beneficial for growth in sub-lethal levels of blue light (O'Donoghue *et al.* 2016). Taken together, these results suggest that there are two effects at play, one being the negative effect of SigB on growth rate in the presence of low doses of blue light, and the other being the protective effect on stress resistance genes which confers resistance to high doses of blue light. Despite evidence suggesting that growth inhibition of *L. monocytogenes* by blue light is due to reactive oxygen species (ROS) (O'Donoghue *et al.* 2016), exposure of *L. monocytogenes* to blue light does not alter the transcription of the *sod* or *kat* genes thought to be involved in tolerance to ROS (Ondrusch and Kreft 2011).

1.5.5 Bile stress

Upon exit from the stomach and entrance to the gastrointestinal tract, *L. monocytogenes* will come into contact with bile. Bile is secreted from the liver and is composed of water with dissolved electrolytes, lipids, inorganic anions, vitamins, proteins, peptides, amino acids and heavy metals (Boyer 2013). *L. monocytogenes* is able colonise and survive in the human gall bladder, leading to biliary tract listeriosis and demonstrating a high level of bile tolerance (Charlier *et al.* 2014). When tested *in vitro*, *L. monocytogenes* was able to grow in the presence of up to 30% bovine bile in BHI broth, despite the physiological bile concentration being predicted at 0.3% in bovines (Begley *et al.* 2002). Interestingly, pre-exposure to low pH (5.5) induces a protective effect against bile in *L. monocytogenes* (Begley *et al.* 2002), indicating a physiological adaptation of this pathogen to improve survival within the gastrointestinal tract of the host.

Proteomic analysis of *L. monocytogenes* in the presence of bile identified differential expression in proteins involved in cell envelope and cellular processes, metabolism and stress response and repair proteins (Payne *et al.* 2013). Deletion of either *sigB* or *prfA* from the genome reduces the ability of *L monocytogenes* to tolerate bile stress *in vitro* (Begley *et al.* 2005), suggesting that both of these transcriptional regulators play a role in bile tolerance. The *bsh* gene of *L. monocytogenes* encodes a bile salt hydrolase enzyme, and is under the control of both PrfA and SigB (Zhang *et al.* 2011). The use of Δ*prfA,* Δ*sigB* and Δ*prfAsigB* mutants demonstrated that the deletion of either of these transcriptional regulators had a slight effect on the ability of *L. monocytogenes* to grow in the presence of 3% bile salts, but the deletion of both transcriptional regulators almost abolished growth under these conditions (Zhang *et al.* 2011). When examined under acidic conditions (pH 5.5), transcriptomic analysis identified upregulation of the PrfA regulation and downregulation of the SigB regulon (Guariglia-Oropeza

et al. 2018). The deletion of *bsh* from the genome significantly reduces the persistence of *L. monocytogenes* in the guinea pig gastrointestinal tract (Dussurget *et al.* 2002), indicating a role for Bsh in the early stages of infection.

A second virulence factor of *L. monocytogenes* involved in bile stress tolerance is BilE, a bile exclusion system under the control of PrfA (Sleator *et al.* 2004). The *bilE* locus contains two open reading frames, *bilEA* and *bilEB*, which together encode a bile permease that is required to export bile from the cell and prevent intracellular bile accumulation (Sleator *et al.* 2004). Despite initially being characterised as an ABC transporter (Glaser *et al.* 2001), the BilE system was confirmed as a bile transporter when it was expressed in *Bifidobacterium breve* and *Lactococcus lactis,* increasing the tolerance of both organisms to bile (Watson *et al.* 2008). However, determination of the crystal structure of the BilE C-terminus found distinct differences in its structure compared to that of structured homologs (Ruiz *et al.,* 2016). While BilE has a known role in bile tolerance, the novel crystal structure of the C-terminus suggests that it may have an uncharacterised role in bile resistance rather than being a bile efflux pump.

1.5.6 Ethanol stress

Ethanol is a common antimicrobial agent used in a variety of industries. Unlike the response of *L. monocytogenes* to many other stresses, the response of the bacterium to ethanol stress is at least partially independent of SigB (Ferreira *et al.* 2001). When wild-type and Δ*sigB* strains were pre-exposed to sublethal levels of ethanol prior to exposure to a lethal dose of ethanol, both strains showed an equal adaptive response, suggesting that the response is independent of SigB (Ferreira *et al.* 2001). Conversely, a 5 min incubation of *L. monocytogenes* in BHI broth supplemented with 16.5% (v/v) ethanol resulted in a significant increase in the transcription of the SigB-

dependent gene *opuCA* (Chaturongakul and Boor 2006). This paradox may be explained by the higher level of ethanol leading to the activation of a general stress response rather than a response targeted to ethanol stress.

The LisRK and CesRK two-component systems have both been implicated in the response of *L. monocytogenes* to ethanol (Cotter *et al.* 1999, Kallipolitis *et al.* 2003). Growth experiments in the presence of 5% ethanol demonstrated a role for LisRK in ethanol tolerance, with the Δ*lisK* mutant able to grow in the presence of this concentration of ethanol which was bacteriostatic for the wild-type (Cotter *et al.* 1999). This ethanol resistance phenotype of the Δ*lisK* mutant can be reversed by overexpressing *lisR* (Cotter *et al.* 2002). As ethanol stress is thought to act upon the cytoplasmic membrane, it is hypothesised that the LisRK two-component system regulates the composition of this membrane (Hill *et al.* 2002).

Deletion of either *cesR* or *cesK* from the genome of *L. monocytogenes* results in increased ethanol tolerance phenotype (Kallipolitis *et al.* 2003). The deletion of a gene under the control of the CesRK two-component system, *orf2420,* also resulted in an ethanol resistant phenotype (Kallipolitis *et al.* 2003). Upon exposure to ethanol, transcription of *orf2420* is strongly induced (Kallipolitis *et al.* 2003), suggesting that transcription of this peptide is required for the response of *L. monocytogenes* to ethanol. The transcription of *lmo0443, lmo1416, lmo2210* and *lmo2812* is also induced in a CesRK-dependent manner upon ethanol exposure (Gottschalk *et al.* 2008). When deletion mutants of these four genes were constructed, the Δ*lmo1416* mutant showed an ethanol tolerant phenotype similar to that of the Δ*cesR* and *ΔcesK* mutants, however the Δ*lmo0443,* Δ*lmo2210* and Δ*lmo2812* mutants showed an ethanol sensitive phenotype similar to the wild type (Gottschalk *et al.* 2008).

Exposure of *L. monocytogenes* to ethanol has been shown to induce surface attachment at 10, 20 and 30°C, but not at 37°C (Gravesen *et al.* 2005). At 10°C, this phenotype was independent of SigB, however, at 20°C, the Δ*sigB* mutant showed a significant decrease in surface attachment compared to the wild-type (Gravesen *et al.* 2005). Similarly, the Δ*cesK* mutant showed significantly higher levels of attachment compared to the wild-type at 10°C, but not at 20°C (Gravesen *et al.* 2005).

1.5.7 Biofilm formation and motility

Biofilms are often found on surfaces in food processing environments and are formed by aggregations of cells held together by an extracellular matrix, often composed of polysaccharide. A study into the effects of surface roughness on the adhesion and viability of *L. monocytogenes* found that biofilms form more readily on rougher surfaces, however cell viability increases on smoother surfaces (Silva *et al.* 2008). A similar study assessed the role of growth medium and temperature in biofilm formation, found that there was a significant impact on biofilm formation by growth medium, but only a minimal effect of growth temperature (Moltz and Martin 2005). Two papers confirm a role for SigB in biofilm formation (Lemon *et al.* 2010, van der Veen and Abee 2010). The study by van der Veen and Abee (2010) identified significant levels of SigB activity during biofilm formation under static and continuous flow conditions, and also a significant decrease in biofilm formation under these conditions in the Δ*sigB* mutant compared to the wild-type (van der Veen and Abee 2010). In agreement with van der Veen and Abee (2010), Lemon *et al.* (2010) confirmed a requirement for SigB in biofilm formation at 30°C only. Likewise, work in *S. aureus* and *S. epidermidis* has identified SigB as one of several regulators involved in biofilm formation (Knobloch *et al.* 2001, 2004, Lauderdale *et al.* 2009, Abee *et al.* 2011). Investigations into the role of SigB in *B. subtilis* biofilm formation have shown mixed results. In one study, the *ΔsigB* mutant was

unable to form a biofilm (Nagórska *et al.* 2008), while a second study found that while Δ*sigB* strain could form a biofilm, but the cells showed an increase in stress sensitivity and a reduction in cell viability (Bartolini *et al.* 2019). The results of these studies are indicative of a variable, and possibly temperature-dependent role for SigB in biofilm formation.

An earlier study found that motility via the flagella is an absolute requirement for biofilm formation, with mutants that are defective in flagella formation unable to form biofilms in microtitre plates (Lemon *et al.* 2007). SigB is a negative regulator of motility through its regulation of the transcriptional repressor of motility genes, MogR (Toledo-Arana *et al.* 2009). In contrast to the study by Lemon *et al.* (2007), the study by Todhanakasem *et al.* (2008) found that the loss of flagella inhibited the initial attachment of cells to the surface, but resulted in hyperbiofilm formation when conducted in flow cells. However, in agreement with Lemon *et al.* (2007), Todhanakasem (2008) identified a requirement for flagella in biofilm formation in microtitre plates. When the results of these studies are considered together, they indicate a role for SigB in biofilm formation that depends on the environmental conditions encountered.

It has been known that *L. monocytogenes* only expresses flagella at temperatures below 37°C for over 30 years (Peel *et al.* 1988). More recent studies have shown that motility is repressed at 37°C by MogR binding upstream from a flagellin-encoding gene, *flaA,* and inhibiting transcription (Gründling *et al.* 2004). At temperatures ≤30°C, the transcriptional repressor activity of MogR is inhibited by an anti-repressor, GmaR, that complexes with MogR and prevents binding to its DNA target sites (Shen *et al.* 2006). At physiological temperatures, GmaR undergoes a conformational change, inhibiting complex formation with MogR, therefore leaving MogR free to bind to DNA target sites and repress the transcription of motility genes (Kamp *et al.* 2011) (Figure 1.3).

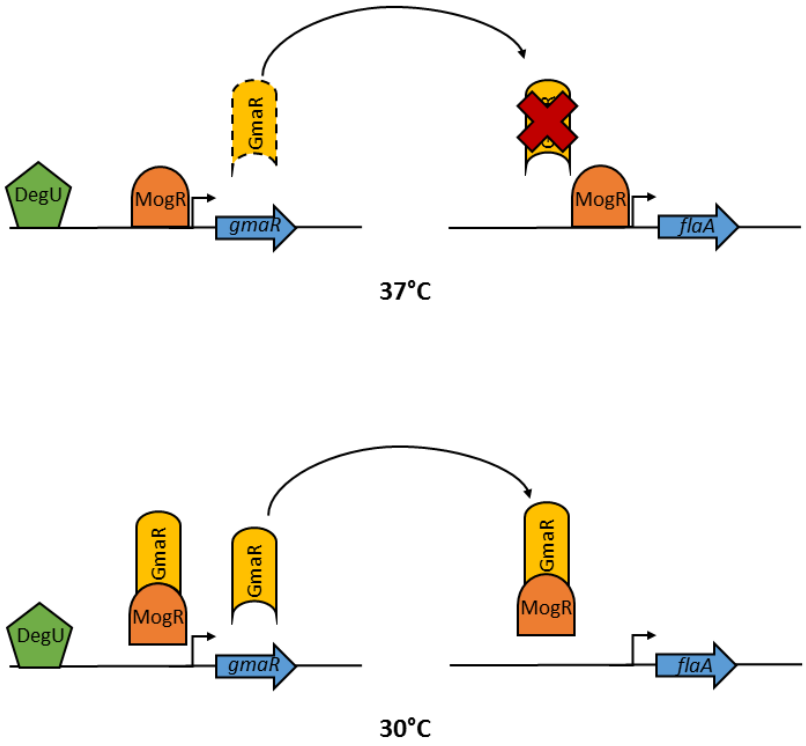

Figure 1. 3 Temperature regulation of *L. monocytogenes* flagella

At 37°C *L. monocytogenes* is immotile due to MogR repressing the transcription of the *flaA,* encoding a flagellar protein. Low levels of GmaR are translated, but degrade before they are able to bind to MogR and remove it from the transcriptional start site of *flaA*. At 30°C, GmaR is transcribed and translated, enabling it to bind to MogR and increase transcription of *gmaR* and *flaA*. Figure adapted from Kamp *et al.* (2011).

A previous study has shown that the Δ*sigB* mutant has increased motility at 37°C compared to the wild-type (Raengpradub *et al.* 2008). The transcriptional study by Toledo-Arana *et al.* (2009) identified a SigB promoter upstream of *mogR,* that enables the transcription of three genes involved in flagellar biosynthesis, *lmo0675, fliP* and *fliQ.* Therefore, the increased motility of the Δ*sigB* mutant is likely to be because *mogR* is not expressed. In the absence of SigB motility is increased, suggesting biofilm formation should be increased if motility is an absolute requirement for biofilm formation. Therefore, the reduction in biofilm formation in the Δ*sigB* mutant suggests that either dysregulation of motility negatively impacts

biofilm formation, or, more liklely, that SigB is required for a role other than regulation of motility in biofilm formation.

1.6 Bacterial blue light sensing systems

Within non-phototrophic bacteria, the three domains for sensing blue light include Light, Oxygen or Voltage (LOV) domains, Blue-Light-utilising flavin adenine dinucleotide (FAD) (BLUF) domains, and Photoactive Yellow Protein (PYP) cryptochrome domains (Jung *et al.* 2005).

1.6.1 Light, Oxygen or Voltage domains

The characterisation of Lmo0799 as a blue light sensing protein was initially based on its similarity to the blue light sensing protein, YtvA, in *Bacillus subtilis* (Ondrusch and Kreft 2011). Through sequence comparison, it has been noted that YtvA and Lmo0799 share 54% homology (Chan *et al.* 2013), and several amino acids that are required for the functioning of YtvA as a blue light sensor are conserved in Lmo0799 (Ondrusch and Kreft 2011). These conserved amino acids include the cysteine residue at positions 62 and 56 in YtvA and Lmo0799, respectively, that is essential for photoadduct formation in response to light (Gaidenko *et al.* 2006, O'Donoghue *et al.* 2016).

The first study to characterise YtvA as a blue light sensor identified a light, oxygen, voltage (LOV) domain upstream from the STAS domain that shared a high level of homology with plant phototropins (Losi *et al.* 2002). LOV domains are characterised by the ability of environmental factors to alter their redox potential (Huala *et al.* 1997), and are part of the larger family of signalling molecules called Per-Arnt-Sim (PAS) domains (Taylor and Zhulin 1999). LOV domains are able to regulate kinase activity in response to

excitation by blue light via their reversible binding of flavin domains (Huala *et al.* 1997). PAS domains are composed of three parts: (1) the PAS core which is composed of A_β, B_β, C_α, D_α and E_α, (2) the helical connector known as F_α, and (3) the β-scaffold formed of the three β-sheets G, H and I (Taylor and Zhulin 1999). The modelling of YtvA and Lmo0799 shows that the conserved cysteine residues at position 62 and 56, respectively, are located in a pocket on the N-terminus of the Eα strand (Ondrusch and Kreft 2011).

Investigations into the photochemistry of the YtvA LOV domain showed that, like the characterised LOV domains found in plants, the YtvA LOV domain was able to bind a flavin mononucleotide (FMN) as a chromophore (Losi *et al.* 2002). FMN is the product of the phosphorylation of riboflavin by riboflavin kinase (Wishart *et al.* 2018). Within the FMN is a carbon atom, C(4a), that forms a reversible covalent adduct with the conserved cysteine residue of the LOV domain upon irradiation with blue light (Christie 2007). The conserved cysteine residue in YtvA (Cys62) is critical for the activation of SigB in response to blue light irradiation, and its alteration to either a serine or alanine residue inhibits SigB activation in response to blue light irradiation (Ávila-Pérez *et al.* 2006, Gaidenko *et al.* 2006). Likewise, the conversion of the conserved cysteine residue in Lmo0799 (Cys56) (Ondrusch and Kreft 2011) to an alanine residue inhibits the formation of ringed colonies in oscillating cycles of light and dark (O'Donoghue *et al.* 2016), suggesting that it is also critical for the activation of SigB in *L. monocytogenes* in response to blue light irradiation.

1.6.2 Blue-Light-Utilising flavin adenine dinucleotide domains

Blue-Light-Utilising flavin adenine dinucleotide (BLUF) domains are blue light sensing domains that rely on FAD chromophore binding, and are found in, but not exclusively, *Rhodobacter sphaeroides* (Gomelsky and Klug 2002), *Klebsiella pneumoniae* (Barends *et al.* 2009) and *E. coli* (Gomelsky and Hoff

2011). BLUF proteins can be divided into two categories: complex and short. Short BLUF proteins are accompanied by around 50 amino acids that form no functional protein at the C-terminal end of the protein, while complex proteins have sensor and effector molecules added to the C-terminal end rather than just amino acids (Jung *et al.* 2005).

The AppA protein of the photosynthetic bacterium *R. sphaeroides* was the first BLUF protein identified to have a domain capable of binding FAD (Gomelsky and Klug 2002). In the absence of blue light, AppA is able to bind to PpsR, a regulatory protein able to repress the expression of genes involved in the production of bactriochlorophyll and carotenoid, light harvesting and respiration, preventing PpsR from repressing the expression of these genes (Masuda and Bauer 2002). Upon excitation by blue light, AppA is no longer able to bind PpsR, and therefore acts as a repressor of photosynthetic gene expression as PpsR is free to act as a repressor (Masuda and Bauer 2002).

In *E. coli,* YcgF is a BLUF-EAL protein that acts as an antagonist of the repressor protein YcgE (Tschowri *et al.* 2009). This is unusual for a BLUF-EAL protein, as usually the EAL domain cleaves cyclic-di-GMP (Gomelsky and Hoff 2011). Upon its activation by blue light, YcgF binds to YcgE, inhibiting its repressor activities and resulting in the strong upregulation of six genes involved in the RCS two-component system (Tschowri *et al.* 2009). Upregulation of these genes result in increased acid resistance and biofilm formation and inhibition of curli formation (Tschowri *et al.* 2009). It is thought that upregulation of these genes may provide *E. coli* with a survival advantage when inhabiting an aquatic environment (Tschowri *et al.* 2009). As light will exist in this environment, light may act as a signal to the bacterium that is in an environmental as opposed to an intracellular niche.

1.6.3 Photoactive Yellow Proteins

A photoactive yellow protein (PYP) is a third type of bacterial blue light-sensing protein, first discovered in *Ectothiorhodospira halophila,* a phototropic bacterium (Mcree *et al.* 1986). When *E. halophila* is exposed to light at wavelengths of 400 to 500 nm, the bacteria exhibit a photophobic response (Sprenger *et al.* 1993), correlating with the peak absorption of the PYP protein at 446 nm (Mcree *et al.* 1986). These results suggest that the presence of the PYP protein is required to initiate a protective response to blue light exposure in this photosynthetic organism. Similarly to LOV domains, the chromophore, in this case 4-hydroxy-cinnamate (Hoff *et al.* 1996), of the *E. halophila* is covalently bound to Cys69 of the protein (Van Beeumen *et al.* 1993).

1.6.4 The effects of light on bacteria other than *L. monocytogenes*

The electromagnetic spectrum ranges from short wavelength, high frequency gamma rays, through the visible range of the spectrum, to long wavelength, low frequency radio waves (Fig. 1.4) (Chan and Almutairi 2016). Ultraviolet light has been used for a long time for the sterilisation of products and materials, but it is only recently that the therapeutic potential of blue light has been investigated. The ultraviolet range of the spectrum spans from 10 to 400 nm wavelengths, while the visible range spans from 400 to 700 nm wavelengths (Gwynne and Gallagher 2018). Photodynamic inactivation can use light either alone or in combination with a drug that reacts with the light to produce reactive oxygen species, increasing the bactericidal potential of the therapy (Wainwright *et al.* 2017).

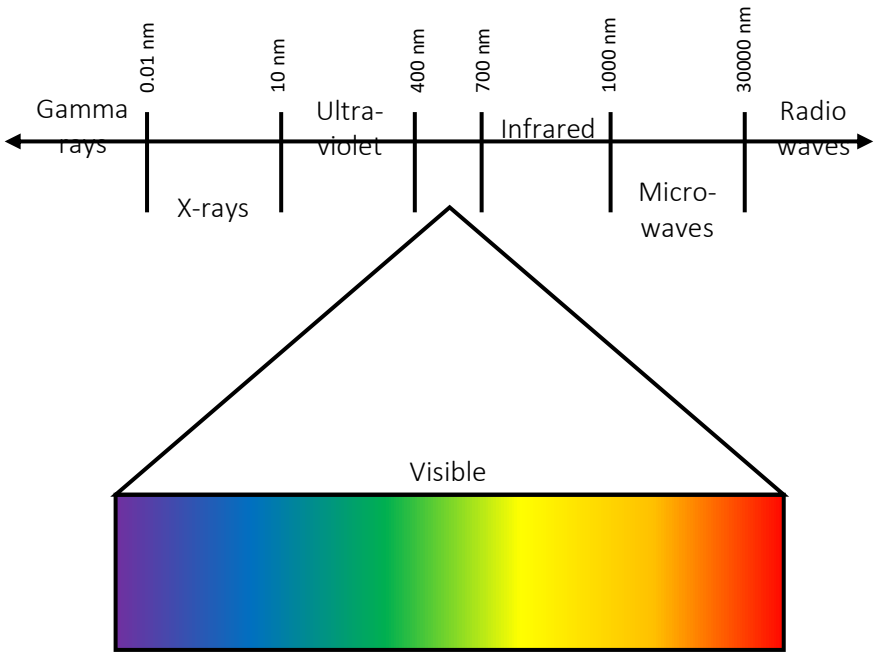

Figure 1. 4 The electromagnetic spectrum

The electromagnetic spectrum ranges from short wavelength, high energy gamma rays to long wavelength, low energy radio waves, with only the visible range being visible to the naked eye.

The ability of low intensity (7.2 J/cm^{-2}) blue light to produce reactive oxygen species has been proven (Lavi *et al.* 2004). The idea that ROS are the primary bactericidal mechanism of photodynamic inactivation is backed up by evidence that the reversal of growth inhibition in the presence of low-intensity blue light can be induced by the addition of ROS scavengers to the medium (O'Donoghue *et al.* 2016). This is supportive of previous work showing that the presence of ROS-scavengers induce a protective response to high-intensity (750 J/$^{cm-2}$) violet/blue light (Endarko *et al.* 2012) and also that oxygen depletion reduces the bactericidal properties of light exposure (Maclean *et al.* 2008).

The multiple cellular targets of ROS reduces the ability of the cells to develop resistance, a common problem associated with the use of traditional antimicrobials (Wainwright *et al.* 2017). While this is a newly emerging

antimicrobial therapy, photodynamic inactivation has been trialled *in vivo* against *Streptococcus mutans* causing dental caries in children (Bargrizan *et al.* 2018), and *Helicobacter pylori* gastric infections (Faraoni *et al.* 2018). Murine studies have also identified a role for photodynamic inactivation against *S. aureus* and *E. coli* in wounds (Hamblin *et al.* 2007). These *in vivo* trials indicate that blue light may be an effective method of reducing dental carries and treating gastric and wound infections through inactivation of the bacteria.

1.7 Two-component systems

Two-component systems are composed of a response regulator domain and a histidine kinase domain (Figure 1.5) (Hoch and Silhavy 1995). Upon detection of a signal, the conserved histidine residue within the histidine kinase domain is phosphorylated by ATP, and these phosphoryl groups are transferred to a conserved aspartic acid residue within the response regulator domain (Bourret *et al.* 2010). The transfer of the phosphoryl group from the sensor kinase to the response regulator induces a conformational change in the response regulator, thereby activating the response regulator and enabling it to induce a response (Gao and Stock 2009).

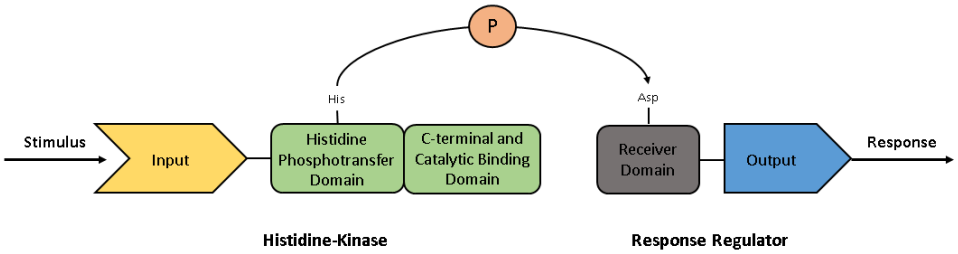

Figure 1. 5 Structure of a two-component system.

A typical two-component system is composed of histidine-kinase domain and a response regulator. The histidine-kinase domain is made up an input terminal coupled to a histidine phosphotranser domain and a C-terminal and catalytic-binding domain. The perception of a stimulus is relayed through the input domain and leads to autophosphorylation of a histidine residue, resulting in the transfer of a phosphoryl group to the response regulator. The response regulator is composed of a receiver domain, which receives the signal from the histidine-kinase domain, and an output domain which elicits a response. Figure adapted from Gao and Stock (2009).

1.7.1 LisRK

The LisRK two-component system of *L. monocytogenes* was first identified by Cotter *et al.* (1999) and shown to play a role in acid and ethanol tolerance, as well as virulence. The use of a Δ*lisK* mutant, lacking the sensor kinase co1mponent, identified a growth phase-dependent acid resistant phenotype for the mutant, as well as an increased growth rate in the presence of 5% ethanol (Cotter *et al.* 1999). In the absence of *lisK,* the bacteria show significantly increased sensitivity to a variety of antibiotics, including cephalexin and cefuroxin, as well as increased resistance to nisin (Cotter *et al.* 2002). When the Δ*lisK* mutant was complemented to overexpress *lisR*, the bacteria reverted to a nisin-sensitive phenotype in line with that of the wild-type (Cotter *et al.* 2002).

The LisRK two-component system has been shown to regulate the transcription of several genes, including the HtrA-like serine protease, *htrA* (Stack *et al.* 2005). The HtrA protein of *L. monocytogenes* is required for resistance to salt, temperature and hydrogen peroxide stress (Wonderling *et al.* 2004), as well as for resistance to oxidative stress within the intracellular environment and virulence within the murine infection model (Wilson *et al.* 2006). The hypothesised role for HtrA in virulence is the correct folding of secreted proteins under PrfA control that are required for cellular invasion and escape from the host cell vacuole (Ahmed and Freitag 2016).

1.7.2 CesRK

CesRK is a second two-component system of *L. monocytogenes* that has been shown to be involved in the tolerance of *L. monocytogenes* to both ethanol and cell-wall acting antibiotics (Kallipolitis *et al.* 2003). The presence of ethanol, but not cell-wall acting antibiotics has been shown to induce the expression of *orf2420,* a CesRK-dependent gene (Kallipolitis *et al.* 2003). Upon exposure to isopropanol, *L. monocytogenes* upregulates the expression of several genes, four of which, *lmo0443, lmo1416, lmo2442* and *lmo2812*, are under the control of CesRK (Gottschalk *et al.* 2008). The construction of deletion mutants for each of these genes identified roles for *lmo0443* and *lmo2812* in ethanol tolerance as both deletion mutants showed a significantly reduced growth rate compared to the wild-type in the presence of ethanol, and a role for *lmo1416* in resistance to β-lactam antibiotics as the deletion mutant showed a significant increase in sensitivity to cefuroxime and ampicillin compared to the wild-type (Gottschalk *et al.* 2008). When the transcription of these genes was measured after exposure to environmental stress, all four genes were upregulated in the presence of ethanol or salt (Nielsen *et al.* 2012).

In a study concerning the relationship between ethanol and biofilm formation, the CesRK two-component system was shown to regulate the biofilm formation of *L. monocytogenes* in the presence of ethanol, along with SigB (Gravesen *et al.* 2005). Growth experiments in the presence of cefuorixime, sodium chloride, bile salts and SDS identified a role for *cesR* in growth in the presence of cefuorixime and sodium chloride, but not bile salts or SDS (Nielsen *et al.* 2012). Taken together, these studies suggest that CesRK is required for osmotolerance and resistance to cell wall stresses.

1.8 RNAseq as a transcriptomic tool

With the first published studies in 2008 (Nagalakshmi *et al.* 2008), RNAseq provided an updated transcriptomic method compared to microarrays and cDNA sequencing, allowing for high throughput and accurate sequencing with lower costs and a relatively small quantity of starting material required (Wang *et al.* 2009). After RNA is extracted from a sample, the strands are fragmented into pieces of ~200 nt in length then converted to cDNA (Mortazavi *et al.* 2008). From the sequencing of these strands, a molecular library can be constructed of individual reads, which are then either mapped to a reference genome or assembled *de novo* (Mortazavi *et al.* 2008).

In recent years, RNA seq technology has been adapted and combined with additional technologies to create specialised approaches suited to a range of applications which will be briefly discussed in this section (extensively reviewed in Saliba *et al.*, 2017). MAP seq combines the use of MS2 affinity purification, where a sRNA of interest is tagged with an MS2 aptamer which enables it to be purified with its target, with RNA seq to enable the identification of sRNA targets (Lalaouna *et al.* 2015, 2018). Also useful in determining the targets of sRNAs, GRIL seq identifies the targets of sRNAs by ligating sRNA-mRNA duplexes with RNA ligase prior to sequencing (Han

et al. 2017), enabling calculation of mRNA levels as well as sequencing (Saliba *et al.* 2017). The co-immunoprecipitation of a sRNA with its RNA-binding protein prior to sequencing forms the basis for RIP seq (Chao *et al.* 2012), enabling target prediction of a sRNA. Overall, these new RNA seq technologies contribute to identifying the targets of sRNAs as well as determining the levels of RNA and changes in transcription in varying environmental conditions.

The method of RNA seq utilised in a particular research project is determined by the aim and scope of the study. For example, a study that only aimed to investigate the effects of a treatment on the expression of the known SigB regulon would most likely use targeted RNA seq. Targeted RNA seq involves the use of either bioatinylated probes able to bind to specific fragments of cDNA or RNA, or primers that are specific to genes of interest (Hrdlickova *et al.* 2017). However, a study that was interested in the transcriptional changes of individual cells within a population would most likely use single-cell RNA seq (scRNA seq).

1.9 Project Aims

The role of SigB in activating the general stress response of *L. monocytogenes* has been well studied, but the roles of individual RsbR paralogues in stress sensing has not yet been elucidated. The exception to this is Lmo0799, which has been characterised as a blue light sensing protein that is required for the activation of the general stress response in response to blue light exposure (Ondrusch and Kreft 2011, Tiensuu *et al.* 2013, O'Donoghue *et al.* 2016). The aim of this project was to investigate the role of SigB and the individual RsbR paralogues in the sensing and resistance to environmental stresses.

The first objective of this study was to investigate the relationship between environmental factors and the tolerance of *L. monocytogenes* to blue light (Chapter 3). Upon identifying potential influencing factors, the role of SigB in altering the tolerance of *L. monocytogenes* to blue light in response to these factors was to be assessed. Finally, the ability of various substances commonly found in food processing environments to induce cross-resistance to blue light in *L. monocytogenes* was assessed.

The second objective of this study was to determine the transcriptional effect of blue light in *L. monocytogenes*, and to investigate the role of SigB and Lmo0799 in this response (Chapter 4). Through the data generated in this investigation, the study aimed to identify potential molecular roles for SigB and Lmo0799 Cys56 in light sensing and resistance, and to determine requirement for Lmo0799 Cys56 to activate the general stress response in response to blue light exposure was assessed.

The third objective of the study was to investigate the roles of RsbR and its paralogues as environmental stress sensors (Chapter 5). By generating single knockout mutants for each of the paralogues, the study aimed to assess the role of each of the paralogues in sensing and responding to various environmental stresses. Finally, it is unknown how the generation of such mutants influences the composition of the stressosome complex, so Western blotting was utilised to measure the levels of RsbR and RsbS in each of the mutants.

Finally, the fourth objective of this study was to investigate the potential role for SigB in the growth of *L. monocytogenes* on several carbon sources (Chapter 6). The first goal of the study was to elucidate whether SigB activation is required for *L. monocytogenes* to grow in the presence of individual carbon sources, and the second was to assess the role of carbon

sources in influencing the resistance of *L. monocytogenes* to commonly utilised methods of bacterial inactivation in the food processing industry.

Overall, the results from this study provide fundamental information on the stress physiology of an important food-borne pathogen, including insights that are of relevance to both the food processing and the pharmaceutical industries.

Chapter 2: Materials and Methods

2.1 Bacterial strains and plasmids

2.1.1 Bacterial Strains

All of the bacterial strains in this study (Table 2.1) were streaked onto agar plates from permanent freezer stocks. Permanent freezer stocks were generated by resuspending 5 mL of an overnight culture in 1 mL appropriate media supplemented with 7% (v/v) dimethyl sulphoxide (Sigma-Aldrich) and stored at -80°C in a 2 mL cryovial. A small amount of the permanent freezer stock was streaked onto agar, incubated at 37°C overnight then stored at 4°C for up to 1 month. Colonies were selected from these agar plates for all experiments.

Table 2. 1. Strains used in this study.

COB Strain Number	Strain	Genotype	Source
261	*Listeria monocytogenes*	*L. monocytogenes* EGDe wild-type	Cormac Gahan
262	*Listeria monocytogenes*	*L. monocytogenes* EGDe Δ*sigB*	Cormac Gahan
491	*Listeria monocytogenes*	*L. monocytogenes* EGDe WT integrated pKSV7-p2230-egfp1 confirmed COB688/689	Utratna *et al.* (2012)
495	*Listeria monocytogenes*	*L. monocytogenes* EGDe ΔsigB integrated pKSV7-p2230-egfp1 confirmed COB688/689	Utratna *et al.* (2012)
610	*Listeria monocytogenes*	*L.monocytogenes* EGDe with C56A replacement at *lmo0799* gene strain A	Beth O'Donoghue
644	*Listeria monocytogenes*	*L. monocytogenes* EGDe Δ*lmo0799*	Tiensuu *et al.* (2013)
672	*Listeria monocytogenes*	*L. monocytogenes* EGDe Δ*rsbR*	Beth O'Donoghue
677	*Listeria monocytogenes*	*L.monocytogenes* EGDe Δ*prfA*	Jörgen Johansson
702	*Escherichia coli*	*E. coli* pMAD Δ*lmo1642* transformant	Beth O'Donoghue
703	*Escherichia coli*	*E. coli* pMAD Δ*lmo1842* transformant	Beth O'Donoghue
712	*Listeria monocytogenes*	*L. monocytogenes* EGDe Δ*lmo0161*	Beth O'Donoghue
888	*Listeria monocytogenes*	*L. monocytogenes* EGDe Δ*lmo1642*	This study
889	*Listeria monocytogenes*	*L. monocytogenes* EGDe Δ*lmo1842*	This study
934	*Listeria monocytogenes*	*L. monocytogenes* LO28	Kallipolitis and Ingmer (2001)
936	*Listeria monocytogenes*	*L. monocytogenes* LO28 Δ*lisR*	Cotter *et al.* (1999)
953	*Listeria monocytogenes*	*L. monocytogenes* LO28 Δ*cesR*	Kallipolitis *et al.* (2003)
954	*Listeria monocytogenes*	*L. monocytogenes* LO28 Δ*cesK*	Kallipolitis *et al.* (2003)

2.1.2 Growth conditions

Unless otherwise specified, all *L. monocytogenes* cultures were grown in BHI broth (Lab M, England) and all *E. coli* cultures were grown in Luria-Bertani (LB) broth (Sigma-Aldrich), inoculated with several colonies from fridge stock agar plates, and incubated at the appropriate temperature, shaking at 80 rpm, for ~16 h. The OD_{600} was measured for all cultures prior to experiments, and cultures that did not have an OD_{600} of between 2.5 and 3.5 were discarded.

2.2 Preparation of culture media

2.2.1 Preparation of BHI, LB and nutrient broth media

BHI, LB and nutrient (NB) broths were prepared according to manufacturers' instructions. To prepare 1 L of medium, 37 g of BHI broth powder, 20 g LB broth powder or 13 g NB powder (Oxoid) were added to 1 L distilled water (dH_2O) in autoclave safe glassware. The media was sterilised by autoclaving at 121°C for 15 min in a Labo Autoclave (Sanyo). The addition of antibiotics to broth was carried out immediately before use. Broth was stored at room temperature for up to 1 month.

2.2.2 Preparation of BHI and nutrient agar

BHI agar was prepared according to manufacturers' instructions. To prepare 1 L BHI agar, 49 g BHI agar powder (Lab M) was added to 1 L dH_2O in autoclave safe glassware. To prepare 1 L NA, 15 g Bacteriological Agar No 2 (Lab M) was added to 1 L of NB, prepared as described in Section 2.2.1, prior

to autoclaving. To prepare motility agar, 3 g Bacteriological Agar No 2 was added to 1 L broth prior to autoclaving. The media was sterilised by autoclaving at 121°C for 15 min. Agar was cooled to 50°C and any necessary antibiotics were added before being poured into petri dishes. Agar plates were stored at 4°C for up to 1 month, with the exception of motility agar plates which were used within 2 days.

2.2.3 Preparation of chemically defined medium

A chemically defined medium (DM) was prepared using a modified method of that described by Amezaga *et al.* (1995). Stock solutions were prepared and stored for up to one month as described in Tables 2.2 to 2.9. From these stock solutions a working solution of DM was prepared and stored at 4°C for up to one month in darkness as described in Table 2.10.

Table 2. 2. Composition and storage conditions for salt solution (100X).

Component	Supplier	Weight per 1000 mL dH_2O (g)
Dipotassium hydrogen orthophosphate	AnalaR	79.90
Sodium dihydrogen orthophosphate	AnalaR	31.00
Ammonium chloride	AnalaR	10.00
Autoclave and store at room temperature.		

Table 2. 3. Composition and storage conditions for magnesium sulphate solution (100X).

Component	Supplier	Weight per 1000 mL dH_2O (g)
Magnesium sulphate	AnalaR	40.00
Autoclave and store at room temperature.		

Table 2. 4. Composition and storage conditions for ferric citrate solution (50X).

Component	Supplier	Weight per 1000 mL dH_2O (g)
Ferric citrate	Sigma-Aldrich	5.00
Autoclave and store at room temperature.		

Table 2. 5. Composition and storage conditions for amino acids solution (100X).

Component	Supplier	Weight per 1000 mL dH_2O (g)
L-leucine	Sigma-Aldrich	10.00
L-isoleucine	Sigma-Aldrich	10.00
L-valine	Sigma-Aldrich	10.00
L-methionine	Sigma-Aldrich	10.00
L-histidine monohydrachloride monohydrate	Sigma-Aldrich	20.00
Arginine monohydrochloride	Sigma-Aldrich	20.00
Heat gently to 45°C on a magnetic stirrer plate to dissolve. Filter sterilise and store at 4°C in darkness.		

Table 2. 6. Composition and storage conditions for cysteine and tryptophan solution (100X).

Component	Supplier	Weight per 1000 mL dH_2O (g)
L-cysteine hydrochloride	Sigma-Aldrich	10
L-tryptophan	Sigma-Aldrich	10
Filter sterilise and store at 4°C in darkness.		

Table 2. 7. Composition and storage conditions for glutamine solution (50X).

Component	Supplier	Weight per 1000 mL dH_2O (g)
L-Glutamine	Sigma-Aldrich	30
Filter sterilise and store at 4°C in darkness.		

Table 2. 8. Composition and storage conditions for vitamin solution (100X).

Component		Weight per 1000 mL dH_2O (g)
Part A		
A-lipoic acid (D-L, 6,8 Thiotic Acid)	Sigma-Aldrich	0.05
Ethanol (70%)	Lennox	200 mL
Part B		
Solution from Part A	Sigma-Aldrich	4 mL
Biotin	Sigma-Aldrich	0.01
Thiamine	Sigma-Aldrich	0.1
Riboflavin	Sigma-Aldrich	0.1
Ethanol (95%)	Lennox	250 mL
Top up to 1000 mL with dH_2O		
Filter sterilise and store at 4°C in darkness.		

Table 2. 9. Composition and storage conditions for trace elements solution (100X).

Component	Supplier	Weight per 1000 mL dH_2O (g)
Part A		
dH_2O	-	800 mL
Sodium hydroxide	AnalaR	6.75
Nitriloacetic acid	Sigma-Aldrich	13.5
Part B		
dH_2O	-	160 mL
Calcium chloride dihydrate	AnalaR	0.55
Zinc chloride	Sigma-Aldrich	0.17
Cupric chloride dihydrate	AnalaR	0.059
Cobaltous chloride 6-hydrate	AnalaR	0.06
Sodium molybdate dihydrate	AnalaR	0.06
Slowly combine and mix solution A and B and make up to 1000 mL with dH_2O. Filter sterilise and store at 4°C in darkness.		

Table 2. 10. Composition and storage conditions for defined medium working stock.

Component	Volume (mL)
dH_2O	810
Salt solution (10 X)	100
Magnesium sulphate solution (100 X)	10
Ferric citrate (50 X)	20
Amino acids solution (100 X)	10
Cysteine and tryptophan solution (100 X)	10
Glutamine solution (50 X)	20
Vitamin solution (100 X)	10
Trace elements	10
Supplement with glucose to 0.4% final concentration	4 g
Store at 4°C in darkness.	

2.2.4 Phosphate buffered saline

Phosphate buffered saline (PBS) was prepared by dissolving one PBS tablet (Sigma-Aldrich) in 200 mL dH_2O, and autoclaving at 121°C for 15 min. This produced a solution with 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride at pH 7.4.

2.2.5 Additions to culture media

2.2.5.1 Antibiotics

The antibiotics used in this study were chloramphenicol (Sigma-Aldrich) and erythromycin (Sigma-Aldrich), both were stored at room temperature prior to dilution. To prepare stock concentrations, 50 mg/mL chloramphenicol, and 10 mg/mL erythromycin were dissolved in 5 mL absolute ethanol. Solutions were filter sterilised and stored for up to 6 months at -20°C.

2.2.5.2 Ethanol

Autoclaved broth was cooled to room temperature and absolute ethanol was added to a concentration of 4% (v/v). Broth was used within one week of supplementation with ethanol.

2.2.5.3 Acid

For assays requiring acidified BHI broth, broth was acidified to pH 5.8 or pH 2.5 with hydrochloric acid (AnalaR) prior to autoclaving. The pH of the broth was measured with a pH probe (Mettler Toledo) before autoclaving, and a small aliquot was measured prior to use in experiments to check the pH. Acidified broth was used within one week.

2.2.5.4 Sodium chloride

For assays requiring BHI broth with sodium chloride, anhydrous sodium chloride (Sigma-Aldrich) was added to BHI broth at a concentration of 0.5 M prior to autoclaving.

2.3 Construction of mutants

2.3.1 Preparation of electrocompetent cells

An overnight culture of *L. monocytogenes* EGDe cells grown at 37°C in BHI broth, shaking at 80 rpm, was diluted 1:100 in 500 mL BHI broth containing 500 mM sucrose (AnalaR) to give a starting OD_{600} 0.01-0.02. Cells were

grown at 37°C, shaking at 80 rpm, to OD_{600} 0.2-0.25, corresponding to 2.5^9 cfu/mL, then 10 µg mL^{-1} ampicillin (Sigma-Aldrich) was added. Cells were incubated for 2 h at 37°C, allowing OD_{600} to reach ~0.4-0.5. Cells were cooled on ice for 10 min, divided between chilled centrifuge pots and centrifuged 5000 x *g* for 10 min at 4°C. Cells were resuspended in 500 mL total volume of sucrose-glycerol wash buffer (SGWB) (10% glycerol (Sigma-Aldrich), 500 mM sucrose, adjusted to pH 7.0 with 100 mM NaOH), centrifuged as before and resuspended in 175 mL total volume of SGWB, centrifuged and resuspended in 50 mL total volume SGWB. Cells were incubated for 20 min at 37°C with 10 µg mL^{-1} lysozyme (Sigma-Aldrich), shaking. Cells were centrifuged at 3000 *x g* for 10 min and washed in 20 mL SGWB, centrifuged as before and resuspended in 2.5 mL SGWB to give a concentration of 5^9 cfu/ml. Fifty µl aliquots were stored immediately at -80°C.

2.3.2 Transformation of plasmid into electrocompetent cells

Two microliters of plasmid preparation excised from *E. coli* pMAD Δ*lmo1642* transformant or *E.coli* pMAD Δ*lmo1842* transformant using a plasmid miniprep kit (Qiagen) were inoculated into 50 µl of electrocompetent cells, swirled with the pipette tip and incubated on ice for 10 min. A negative control of cells inoculated with nuclease-free water was also prepared. The culture was put into an electroporation cuvette and electroporated on a MicroPulser™ (Bio-Rad) on the bacteria, EC2 programme. Four hundred and fifty microliters of BHI sucrose was added to the electroporated culture and the culture was incubated at 37°C for 1.5 – 2 h to allow cell recovery. Each culture was plated onto both BHI and BHI with 2 µg/mL erythromycin agar plates and incubated at 30°C for 2 days until visible colonies were present. Colonies were confirmed to be *Listeria spp.* by PCR targeting *sigB* as described in Section 2.6.1.

2.3.3 Integration of plasmid into transformed cells

BHI broth was prepared in 5 mL aliquots with 2 µg/mL erythromycin, and one colony of each transformant was added into separate tubes. The tubes were incubated at 42°C in a water bath until growth was detected. Once visible growth was detected, 50 µl of culture was inoculated into 5 mL BHI broth with 2 µg/mL erythromycin and incubated at 42°C in a water bath. This was repeated three times. Each time the culture was re-subbed, a 200 µl aliquot was extracted, diluted to 10^{-5} in PBS and 100 µl was plated onto BHI agar with 2 µg/mL erythromycin. The plates were incubated for 24 h at 37°C. Successful integration of the plasmid was confirmed by PCR targeting primers complimentary to the flanking regions of the gene targeted for deletion.

2.3.4 Excision of plasmid from genome

Transformed cells were passaged in BHI broth without antibiotic selection at 30°C and re-passaged every 12 h into fresh BHI broth for 5 days. At each passage, 200 µl was diluted 10^{-7} in PBS and 50 µl was spread onto BHI agar plates with and without 2 µg/mL erythromycin. Plates were incubated for 24 h at 37°C. When colonies were no longer able to grow in the presence of erythromycin, individual colonies from BHI agar plates were replica stabbed onto BHI and BHI with erythromycin. Plates were incubated at 37°C and colonies that grew only on BHI subjected to PCR to confirm both loss of the plasmid and knockout of the wild-type gene.

2.3.5 Whole genome sequencing

2.3.5.1 DNA extraction

Whole genome sequencing was carried out by Microbes NG (University of Birmingham) from DNA extracted from an overnight culture of each strain. DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen) according to manufacturers' instructions. Briefly, a 2 mL aliquot of overnight culture was centrifuged at *5000 x g* for 10 min and the pellet was resuspended in 180 µl enzymatic lysis buffer (Table 2.11). The mixture was incubated for 30 min at 37°C in a water bath, then 25 ul Proteinase K (Qiagen) and 200 µl Buffer AL were added. The mixture was incubated for 30 min at 56°C in a water bath, then 200 µl of absolute ethanol was added. The mixture was pipetted into a DNeasy Mini spin column in a 2 mL collection tube and centrifuged at *6000 x g* for 1 min. The collection tube was replaced, 500 µl Buffer AW1 was added to the spin column, and the spin column was centrifuged at *6000 x g* for 1 min. The collection tube was replaced, 500 µl Buffer AW2 was added to the spin column, and the spin column was centrifuged at *20000 x g* for 3 min. The spin column was transferred to a 1.5 mL Eppendorf tube, and the DNA was eluted in 100 µl nuclease-free water (Ambion) by centrifuging at *6000 x g* for 1 min. The elution step was repeated once using the flow through from the column to maximise DNA yield. DNA was quantified by Nanodrop (ThermoFisher).

Table 2. 11. Composition of enzymatic lysis buffer.

Component	Supplier	Concentration Required
Tris – HCl (pH 8.0)	Sigma-Aldrich	20 mM
EDTA	AnalaR	2 mM
Triton X-100	Sigma-Aldrich	1.2 %
Lysozyme	Sigma-Aldrich	20 mg/mL

2.3.5.2 Analysis of WGS data

In order to identify single nucleotide polymorphisms that may have arisen spontaneously during mutant construction, the *L. monocytogenes* EGDe, *L. monocytogenes* EGDe Δ*sigB, L. monocytogenes* EGDe Δ*rsbR, L. monocytogenes* EGDe Δ*lmo0161, L. monocytogenes* EGDe Δ*lmo0799, L. monocytogenes* EGDe Δ*lmo1642, L. monocytogenes* EGDe Δ*lmo1842,* and *L. monocytogenes* EGDe C56A *lmo0799* strains were sent for WGS. The data was analysed using the *breseq* computational pipeline, comparing the two sets of trimmed reads for each mutant to the assembled and annotated genome sequence of the wild-type.

2.4 Stress survival assays

2.4.1 Temperature-dependent light survival assay

Two overnight wild-type and Δ*sigB* cultures were prepared, and incubated at either 30°C or 37°C in 25 mL BHI broth, shaking in darkness. For experiments using exponential phase cells, fresh broth was inoculated to OD_{600} 0.05 and incubated at either 30°C or 37°C in darkness, shaking, until growth reached OD_{600} ~0.2. Cells were washed in PBS and resuspended in PBS at OD_{600} 1. Three 200 µl aliquots of each strain were put in round-bottomed 96-well plates (ThermoFisherScientific) and were incubated at 30°C for 6 h (exponential phase) or 8 h (stationary phase), either exposed to 35 mW/cm^{-2} 470 nm light using the apparatus shown in Figure 2.1 or kept in darkness. Colony-forming units per millilitre were measured by diluting each strain 10-fold to 10^{-7} in PBS and spotting 10 µl of each dilution in triplicate on to BHI agar plates. Plates were incubated in darkness at 37°C for 48 h, and percent survival was calculated for each strain.

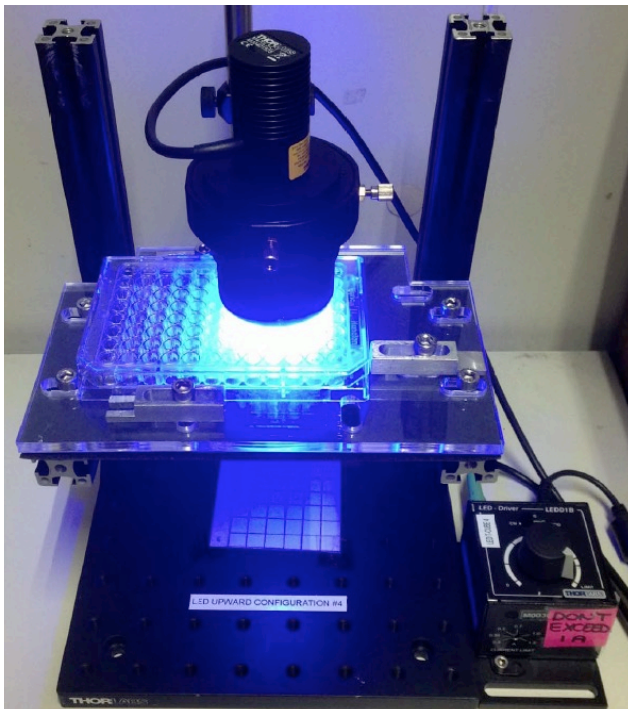

Figure 2. 1 Light apparatus.

The light apparatus used in this study was constructed with a 470 nm LED lamp (ThorLabs) which delivered a maximum uniform irradiance of 35 mW cm^2 and a bandwidth of 25 nm. The light intensity was consistent in an area large enough to cover 16 cm^2 and could be adjusted down to 1 mW cm^2 using a T-cube driver (ThorLabs). The presence of metal screws ensured consistent placing of the 96-well sample plate.

2.4.2 Growth phase-dependent light survival assay

Two overnight cultures were prepared for each strain used in the study, and incubated at 30°C in 25 mL BHI broth, shaking at 80 rpm, in darkness. Fresh broth was inoculated to OD_{600} 0.05 and incubated at 30°C in darkness, shaking, until growth reached OD_{600} ~0.2. Stationary and exponential phase cells were washed in PBS and resuspended in PBS at OD_{600} 1. Three 200 µl aliquots of each strain were put in round-bottomed 96-well plates and were incubated at 30°C for 6 h, either exposed to 35 mW/cm^{-2} 470 nm light or kept in darkness. Colony-forming units per millilitre were measured, and percent survival was calculated for each strain.

2.4.3 Stress-induced light resistance assays

An overnight culture was prepared for each strain used in the study, and incubated at either 30 °C or 37 °C in 25 mL BHI broth, shaking (80 rpm). Fresh broth was inoculated to OD_{600} 0.05 with the overnight culture and incubated at the appropriate temperature, shaking, until growth reached OD_{600} ~0.2. One millilitre aliquots were taken, centrifuged 13000 *x g* for 2 min and the pellet resuspended in BHI broth supplemented to either pH 5.8, 0.5 M NaCl, or 4% (v/v) ethanol. A control culture was resuspended in BHI broth. Cells were incubated for 1 h at the appropriate temperature in darkness, shaking, then centrifuged 13000 *x g* for 2 min. The pellets were washed once and resuspended in PBS. Three 200 µl aliquots of cells incubated in each condition were put in round-bottomed 96-well plates (Thermo Fisher Scientific) and were incubated at 30°C for 5 h either exposed to 35 mW/cm^{-2} 470 nm light or kept in darkness. Colony-forming units per millilitre (cfu/mL) were measured, and percent survival was calculated for each strain.

2.4.4 Alternative carbon sources light resistance assay

An overnight culture of EGDe wild-type was diluted to OD_{600} 0.01 in NB supplemented with 0.4% glucose, glycerol or lactose and incubated for 5 h, shaking at 160 rpm, at 30°C in darkness. At 5 h, the OD_{600} was measured and a 1 mL sample standardised to OD_{600} 0.2 was taken. The cells were washed once in PBS and resuspended to OD_{600} 0.2 in PBS. Two hundred microliter aliquots were made in triplicate in two round-bottomed 96-well plates (ThermoFisher) and incubated for 6 h at 30°C, with one plate exposed to 35 mW cm^{-2} blue (470 nm) light and the other wrapped in aluminium foil. At 0 h, 3 h, 4 h and 5 h, samples were taken for each growth condition, diluted to 10^{-7} in PBS and 10 µl per dilution was plated in triplicate onto BHI agar plates.

Plates were incubated at 37°C for 48 h in darkness and cfu/mL were calculated. The experiment was performed with two biological replicates.

2.4.5 Heat survival assay

The overnight culture of the wild type EGDe strain was diluted in NB with supplements to OD_{600} 0.01, and incubated in flasks shaking (160 rpm) at 30°C. Samples were taken after 5 and 24 hours of incubation. A control was incubated in NB and incubated at 30 °C. The heat inactivation assay was performed at 55°C in a water bath with shaking at 160 rpm (Julabo SW 23, Julabo Labortechnik). Briefly, 200-400 ul of the culture were inoculated into 20 mL of pre-heated NB with supplements, with an equal final cell concentration. Samples were taken at selected time points up to 45 minutes, serially diluted in PBS and plated in triplicate on BHI agar and incubated at 30°C for 48 – 96 h for CFU enumeration. A separate tube containing NB plus supplements held at room temperature was used for the 0 h measurement.

2.4.6 Acid resistance

The overnight culture of the wild type EGDe strain was diluted in NB with supplements to OD_{600} 0.01, and incubated in flasks shaking (160 rpm) at 30°C. The acid inactivation assay was performed at pH 3 and 2 on samples taken after 5 and 24 hours of incubation, respectively, in a heat block at 30°C. Briefly, the cultures were centrifuged, washed twice with PBS and resuspended in 2 mL of acidified NB supplemented with the relevant carbon source (pH adjusted with 1 M HCl), to a final concentration of approximately 8 $\log_{10}$ CFU/mL. A control was incubated in non-acidifed nutrient broth for the duration of the experiment. Samples were taken at selected time points up to 75 minutes, serially diluted in PBS and plated in BHI plates for CFU

enumeration with the spot plating technique, in triplicates. The plates were incubated at 30°C for 2-4 days.

2.5 Protein methods

2.5.1 Buffers

2.5.1.1 Sonication buffer

The reagents used to prepare 50 mL sonication buffer are detailed in Table 2.12. The solution was adjusted to pH 8.0 as required, and autoclaved at 121°C for 15 min.

Table 2. 12. Composition of sonication buffer.

Reagent	Supplier	Quantity (g)
Tris-HCl	Sigma-Aldrich	0.0788
Ethylenediaminetetraacetic acid (EDTA)	AnalR	0.0018
Magnesium chloride	AnalR	0.0508

2.5.1.2 Tricine buffer

The reagents used to prepare the tricine buffer are detailed in Table 2.13. The solution was adjusted to pH 8.45 as required.

Table 2. 13. Composition of tricine buffer.

Reagent	Supplier	Concentration
Tris-base	Sigma-Aldrich	3 M
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich	0.3% (w/v)

2.5.1.3 Running buffer

The reagents used to prepare 1 L running buffer (10X) are detailed in Table 2.14. The solution was adjusted to pH 8.3 as required. Before use, the solution was diluted to 1X in dH_2O.

Table 2. 14. Composition of running buffer (10 X).

Reagent	Supplier	Quantity (g)
Trizma base	Sigma-Aldrich	30.0
Glycine	Sigma-Aldrich	144.0
SDS	Sigma-Aldrich	10.0

2.5.1.4 Cathode buffer

The reagents used to prepare the cathode buffer (10X) are detailed in Table 2.15. Before use, the solution was diluted to 1X in dH_2O.

Table 2. 15. Composition of cathode buffer (10 X).

Reagent	Supplier	Concentration
Trizma base	Sigma-Aldrich	1 M
Tricine	Sigma-Aldrich	1 M
SDS	Sigma-Aldrich	1% (w/v)

2.5.1.5 Anode buffer

To prepare the anode buffer (10X), a 2 M Tris-base solution was prepared and adjusted to pH 8.9. Before use, the solution was diluted to 1X in dH_2O.

2.5.1.6 Transfer buffer

The reagents used to prepare 1 L transfer buffer are detailed in Table 2.16. All of the reagents were dissolved in 400 mL dH_2O and the solution was adjusted to pH 9.2. The solution was topped up to 1 L with dH_2O.

Table 2. 16. Composition of transfer buffer.

Reagent	Supplier	Quantity (g)
Glycine	Sigma-Aldrich	2.927
Trizma base	Sigma-Aldrich	5.81
SDS	Sigma-Aldrich	0.375
Methanol	Sigma-Aldrich	200 mL

2.5.1.7 Tris-buffered saline and TBST

The reagents used to prepare 1 L tris-buffered saline (TBS) (10X) are detailed in Table 2.17. The solution was adjusted to pH 7.6 and autoclaved at 121°C for 15 min. Before use, the solution was diluted to 1X in dH_2O.

Table 2. 17. Composition of tris-buffered saline (10 X).

Reagent	Supplier	Quantity (g)
Trizma base	Sigma-Aldrich	24.2
Sodium chloride	Sigma-Aldrich	80.0

To prepare the 1% TBST solution, 10 mL Tween 20 (Sigma-Aldrich) was added to 990 mL 1X TBS. To prepare the 0.05% TBST solution, 50 mL 1% TBST solution was added to 950 mL 1X TBS.

2.5.2 Protein extraction

The OD_{600} of a 50 mL culture was checked and 10 µg/mL erythromycin was added to stop protein translation. The culture was centrifuged 9000 *x g* for 15 min at 4°C. The pellet was resuspended in 2 mL sonication buffer supplemented with 2 mg/mL lysozyme (Sigma-Aldrich) and incubated for 30 min at 37°C. The culture was centrifuged 9000 *x g* for 15 min at 4°C and the pellet was resuspended in 0.5 mL of sonication buffer supplemented with 1% (vol/vol) protease inhibitor (Sigma-Aldrich). The culture was transferred to a 2 mL screw cap tube containing 0.25 mL 0.50 mm and 0.50 mL 0.10 mm zirconia beads (Thistle Scientific) and vortexed for 10 min, alternating 30 s bead beating and 30 s rest on ice. The preparation was centrifuged 13000 *x g* for 30 min at 4°C to remove cell debris. The supernatant was extracted and stored at 4°C for up to 24 h.

2.5.3 Protein quantification

Protein quantification was carried out using the *DC*™ Protein Assay (BioRad) according to manufacturers' instructions. Briefly, 127 µl Reagent A' was prepared per sample by adding 5 µl DC Reagent S to 250 µl DC Reagent A. Four dilutions of the BSA protein standard were prepared between 0.2 mg/mL and 1.5 mg/mL and 25 µl of standards and samples pipetted into 1.5 mL microfuge tubes. One hundred and twenty-five microliters of RC Reagent I were added to each tube, the tube was vortexed briefly and incubated for 1 min at room temperature. One hundred and twenty-five microliters of RC Reagent II were added to each tube, the tube was vortexed briefly and centrifuged 15000 *x g* for 5 min. The supernatant was discarded and 127 µl of Reagent A' was added to each tube, the tube was briefly vortexed and incubated at room temperature for 5 min until the precipitate was completely dissolved. One millilitre of DC Reagent B was added to each

tube, the tube was vortexed briefly and incubated for 15 min at room temperature. Absorbances were read at 750 nm on a spectrophotometer (ThermoFisher) and protein quantities for each sample were extrapolated from a standard curve generated from the BSA protein standard dilutions. The protein content of each sample was equalised and supplemented with 20% (vol/vol) 5X loading buffer. Samples were heated to 98°C for 10 min and stored at -20°C.

2.5.4 SDS-PAGE

2.5.4.1 Glycine-based SDS-PAGE gel preparation

Glycine-based SDS-PAGE gels were used for the detection of the 27 kDa eGFP and 30 kDa RsbR proteins. The resolving and stacking gels were prepared with 15 % and 4 % (v/v) acrylamide concentrations respectively. The reagents and volumes used to prepare one 1.5 mm gel are listed in Table 2.18. For the resolving gel, 1.5 M Tris-base at pH 8.8 was used, and for the stacking gel, 0.5 M tris-base at pH 6.8 was used. All reagents were stored at 4°C, with the exception of ammonium persulphate (APS; Sigma-Aldrich) and sodium-dodecyl sulphate (SDS; Sigma-Aldrich) which were stored at room temperature and dissolved in dH_2O immediately before use.

Table 2. 18. Composition of glycine-based SDS-PAGE gel.

Reagent	Volume (µl)	
	Resolving Gel	Stacking Gel
dH_2O	1800	3000
30% Acrylamide/bis-acrylamide	4000	670
Tris-base	2000	1250
10% (w/v) SDS	80	50
10% (w/v) APS	80	50
TEMED	8	5
Total	**8 mL**	**5 mL**

Plates were assembled in the casting apparatus (BioRad) and the resolving gel solution was poured to a level 1 cm below the comb. One millilitre dH_2O was added to the top of the gel to produce a straight edge. The gel was left to polymerise at room temperature for 45 min. The water was poured off and the stacking gel was poured on top of the resolving gel. The 10-well comb (BioRad) was inserted into the assembly without air bubbles, and the gel was left to polymerise for 1 h at room temperature.

2.5.3.2 Tricine-based SDS-PAGE gel preparation

For the detection of the 10 kDa RsbS protein, tricine-based SDS-PAGE was used with a 12% acrylamide concentration. The gels were prepared using the method described in section 2.5.3.1, and the reagents used are listed in Table 2.19.

Table 2. 19. Composition of tricine-based SDS-PAGE gel.

Reagent	Volume (µl)	
	Resolving Gel	Stacking Gel
dH_2O	600	2600
30% Acrylamide/bis-acrylamide	4000	540
Tricine buffer (Table 2.13)	3300	1000
50% (v/v) glycerol	2200	-
10% (w/v) APS	34	16
TEMED	6	4
Total	**10 mL**	**4 mL**

2.5.3.3 SDS-PAGE

The SDS-PAGE gels were assembled in the running apparatus (BioRad) and loaded with the standardised protein samples and 5 µl PageRuler™ Plus prestained protein ladder (ThermoFisher). For the glycine based gels, the

cathode reservoir was filled to capacity and the anode reservoir with 300 mL 1X running buffer, while for the tricine-based gels the cathode buffer was filled to capacity with 1X cathode buffer and the anode reservoir was filled with 300 mL 1X anode buffer. The gels were run for 1.5 h at 150 V and 75 mA per gel, to a maximum of 150 mA.

2.5.3.4 Protein transfer and Western blotting

The gels were rinsed in deionised water and soaked in transfer buffer for 10 min. Each gel was added to a stack of blotting paper (BioRad) soaked in transfer buffer and a PVDF membrane that had been charged in methanol for 30 s and rinsed in transfer buffer for 10 min. An additional sheet of blotting paper was applied over the gel and the stack was transferred to a semi-dry blotter (Cleaver Scientific Ltd) and transferred for 1 h at 5 V and 100 mA.

The blot was washed for 3 x 5 min in the appropriate concentration of TBST, then blocked for 1 h in 5% (w/v) skimmed milk (Sigma-Aldrich) prepared in the appropriate concentration of TBST at room temperature. The blot was washed for 3 x 5 min in the appropriate concentration of TBST, then incubated for 16 h with the appropriate primary antibody at 4°C as detailed in Table 2.20. The blot was washed for 3 x 5 min in the appropriate concentration of TBST and incubated with the secondary antibody (mouse anti-rabbit IgG-HRP (Santa Cruz Biotechnology)) for 1 h at room temperature as detailed in Table 2.20. The blot was washed for 3 x 5 min in the appropriate concentration of TBST, and imaged using a chemiluminescent substrate (Amersham) on a LICOR Odyssey®Fc Imaging System (LI-COR Biosciences). Image Studio (LI-COR Biosciences) was used to process and analyse the image.

Table 2. 20. Antibodies used in this study for Western blotting.

Primary Antibody	Primary Antibody Dilution	TBST Concentration	Primary Antibody Dilution Solution	Secondary Antibody Dilution	Secondary Antibody Dilution Solution
Rabbit anti-GFP	1:7500	1 %	5% skim milk	1:3500	5% skim milk
Rabbit anti-RsbR	1:500	0.05%	SignalBoost™ Solution 1 (diluted 1:1 TBS (1X))	1:6500	SignalBoost™ Solution 2 (diluted 1:1 TBS (1X))
Rabbit anti-RsbS	1:1000	0.05%	SignalBoost™ Solution 1 (diluted 1:1 TBS (1X)	1:6500	SignalBoost™ Solution 2 (diluted 1:1 TBS (1X))

2.6 DNA methods

2.6.1 Polymerase Chain Reaction (PCR)

PCR was used during the process of constructing mutants to confirm the presence or absence of a gene. To create a DNA template, a single colony was taken from an agar plate and resuspended in 250 µl sterile dH_2O. A 10 mM solution of deoxyribonucleotide triphosphates (dNTPs) was prepared from the 100 mM stock solutions of dATP, dTTP, dCTP and dGTP (Sigma-Aldrich) in sterile dH_2O. All primers designed in this study were designed using Primer3.

2.7 RNA methods

2.7.1 Sample collection

An overnight culture was prepared for each strain used in the study, and incubated at the appropriate temperature in 5 mL brain heart infusion (BHI) broth (LabOne), shaking (80 rpm). Fifty millilitres of fresh broth were inoculated to OD_{600} 0.05 with the overnight culture and incubated at the appropriate temperature, shaking in darkness, until growth reached OD_{600} ~0.2. In darkness, the culture was divided between two pre-incubated culture flasks, wrapped in aluminium foil, with the top of one covered with a glass petri dish and the other covered with tinfoil, and 5 mL was taken as the 0 h sample and the RNA was stabilised in 10 mL RNAlater (Sigma-Aldrich) in an amber 50 mL tube. Both cultures were placed on magnetic stirrer plates set at 100 rpm, with the culture covered by the glass petri dish illuminated by 0.6 mW cm^{-2} 470 nm light. Five millilitre samples were taken from both the light and dark cultures after 10, 20 and 30 min, and stabilised in 10 mL RNAlater in amber 50 mL tubes. Samples were incubated in the RNAlater for 10 min then 35 mL PBS was added to enable pelleting of the cells during centrifugation. The samples were centrifuged for 5 min at 8500 *x g* at 22°C, the supernatant discarded and the pellets stored at -80°C.

2.7.2 RNA extraction

RNA was extracted using the RNeasy Mini Kit (Qiagen). The sample pellet (collected as described in section 2.6.1) was resuspended in 700 µl RLT buffer and transferred to a 2 mL lysing matrix B tube (MP Bio). The cells were mechanically lysed twice using the FastPrep apparatus at 6 m/s for 40 s, with 5 min rest on ice between each cycle. Samples were centrifuged for

5 min at 12,000 *x g*, and the supernatant was transferred to 700 µl 70% ethanol. Half of the solution was transferred to an RNeasy filter column and centrifuged for 15 s at 8000 *x g*. The flow through was discarded and the step repeated with the remaining solution. The column was washed twice with 500 µl RPE buffer and centrifuged for 15s and 2 min, respectively, at 8000 *x g*. Any remaining wash buffer was removed by centrifuging for 1 min at 8000 *x g*, and the RNA was eluted in 50 µl nuclease-free water (Ambion) by centrifuging for 1 min at 8000 *x g*.

2.7.3 DNase treatment

The RNA was quantified by NanoDrop and then contaminating DNA was removed using TURBO DNA-free (Ambion). Thirty microliters of RNA were added to 3 µl 10X TURBO DNase buffer and 1 µl TURBO DNase. The samples were incubated for 30 min at 37°C, then 2.3 µl DNase Inactivation Reagent and the samples were reincubated for 5 min at room temperature with intermediate mixing. The samples were centrifuged for 2 min at 10,000 *x g* and the supernatant was extracted. The RNA was quantified and the RNA Integrity measured using the Bioanalyzer (Agilent). Only RNA with a RIN of greater than 8 was accepted for further experiments.

2.7.4 Generation of cDNA

Fifteen microliters of RNA were added to 1 µl 10 mM dNTPs (Sigma-Aldrich) and 1 µl random primers (Invitrogen) and incubated 5 min and 65°C then on ice for 1 min. The sample was centrifuged 5 s and 4 µl First Strand Buffer, 1 µl 0.1 M DTT and 1 µl Superscript III was added. The sample was incubated for 5 min at 25°C, 60 min at 50°C and 15 min at 70°C then stored immediately at -80°C. It was assumed that 1 ng/µl of RNA would be converted to 1 ng/µl

cDNA. cDNA concentrations were diluted to 7 ng/µl in nuclease-free water and stored at -20°C for analysis by RT-PCR.

2.7.5 RT-PCR

RT-PCR was carried out using the LightCycler® 480 (Roche). For each pair of primers, a MasterMix large enough for all samples was prepared containing 5 µl 2X QuantiTect SYBR Green (Qiagen), 0.5 µl forward primer, 0.5 µl reverse primer and 1.5 µl nuclease-free water (Qiagen) for each sample. From the MasterMix, 7.5 µl aliquots were made into a 96-well LightCycler® plate (Roche) and 2.5 µl of cDNA standardised to 7 ng/µl diluted 1:10 in nuclease-free water was added. The plates were sealed with transparent sealing film (Roche) and loaded on to the LightCycler® 480. The RT-PCR protocol contained 4 steps: (1) denaturation, (2) cycling, (3) melt curve, and (4) cooling, and is described in Table 2.21.

Table 2. 21. RT-PCR protocol used in this study.

Step	Temperature (°C)	Time
Denaturation	95	15 min
Cycling (45 cycles)	95 53 72	15 s 15 s 30 s
Melt curve	95 53 91	5 s 1 min -
Cooling	40	30 s

Data analysis was carried out using LightCycler® 480 Software Release 1.5.0 (Roche) and Microsoft Excel. Data were analysed using the relative gene expression method following the formula:

$$Relative\ Expression = \frac{Target\ Efficiency^{(Cp\ target\ in\ calibrator - Cp\ target\ in\ test)}}{Reference\ Efficiency^{(Cp\ reference\ in\ calibrator - Cp\ target\ in\ test)}}$$

Primer efficiencies were calculated for each pair of primers using genomic DNA normalised to 70 ng/µl and serially diluted 10-fold to 10^{-3}. Primer efficiency was calculated using using LightCycler® 480 Software Release 1.5.0 (Roche). The RT-PCR primers and their efficiencies used in this study are detailed in Table 2.22.

Table 2. 22. RT-PCR primers used in this study.

COB Primer Number	Primer Sequence	Primer Name	Primer Efficiency	Origin
625	CTATATTTGGATTGCCGCTTAC	sigB-F RT-PCR	1.95	Marta Utratna
626	CAAACGTTGCATCATATCTTC	sigB-R RT-PCR		Marta Utratna
627	CATCGATAAAGGAGAATTTG	opuCA-F RT-PCR	1.77	Marta Utratna
628	CATAACCAATTGAGCGTCTTAG	opuCA-R RT-PCR		Marta Utratna
629	CATATTCGAAGTGCCATTGC	lmo2230-F RT-PCR	2.00	Marta Utratna
630	CTGAACTAGGTGAATAAGACAAAC	lmo2230-R RT-PCR		Marta Utratna
672	TGGGGAGCAAACAGGATTAG	16S-F RT PCR for 16S RNA	1.95	Marta Utratna/ Andreas
673	TAAGGTTCTTCGCGTTGCTT	16S-R RT PCR for 16S RNA		Marta Utratna/ Andreas
883	CGGCCAGAGGAGTAGTCATT	rsbS FWD RT-PCR	1.94	This Study
884	AAGGTCCATCGCCGAAAGTA	rsbS REV RT-PCR		This Study
891	TTTGGCGAAATTCCGGTGATGA	lmo0799 FWD RT-PCR	1.83	This Study
892	AACACACGACCGTTTTCAGCA	lmo0799 REV RT-PCR		This Study
893	TGGGCCGTGTAGATCGTGAA	lmo0161 FWD RT-PCR	1.92	This Study
894	ATGCCTCGACGCCAAGAAGT	lmo0161 REV RT-PCR		This Study
895	ACTGGTGTGAAGAAACGCGAC	lmo1642 FWD RT-PCR	1.83	This Study
896	ACTGTTGCTGAATCGACGAAAGC	lmo1642 REV RT-PCR		This Study
897	ACACCCATGCAAACGAACAAATG	lmo1842 FWD RT-PCR	1.93	This Study
898	GGAGACTCTAATTGCACACCAGA	lmo1842 REV RT-PCR		This Study
899	CGAAGCAGTTAGACTTGTCGGC	rsbR FWD RT-PCR	1.97	This Study
900	CTGTTCGTCAAGGCTAGCGC	rsbR REV RT-PCR		This Study
901	GGCTTTGGAACCGTTGACCA	rsbT FWD RT-PCR	1.98	This Study
902	ACCTGCTCCAAGACCACCTG	rsbT REV RT-PCR		This Study

2.7.6 Genomic DNA extraction

Genomic DNA used to calculate primer efficiencies was extracted from EGDe wild-type cells using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturers' protocol. An enzymatic lysis buffer for the pre-treatment of Gram-positive bacteria was prepared with Tris-EDTA buffer solution pH 8.0 (Sigma-Aldrich), 1.2% Trition® X-100 (Nalgene) and 20 mg/mL lysozyme (Sigma-Aldrich), added immediately prior to use. Wild-type colonies were scraped from a BHI agar stock plate and resuspended in 180 µl enzymatic lysis buffer and incubated for 30 min in a 37°C water bath. Twenty-five microliters of Proteinase K (Roche) and 200 µl Buffer AL were added to the solution, and it was reincubated for 30 min at 56°C. Two hundred microliters of 96% ethanol were added to the solution and vortexed to mix. The mixture was pipetted into a DNeasy spin column and centrifuged for 1 min at 6000 *x g*, with the flow through discarded. Five hundred microliters of Buffer AW1 was added, and the column centrifuged for 1 min at 6000 *x g*, with the flow through discarded. Five hundred microliters of Buffer AW2 was added, and the column centrifuged for 3 min at 20000 *x g*, with the flow through discarded. The spin column was transferred to a clean 1.5 mL Eppendorf tube and 200 µl of Buffer AE was added to the spin column. The column was incubated for 1 min at room temperature then centrifuged for 1 min at 6000 *x g* to elute the DNA. To maximise the yield, the elution process was repeated using the flow through. The DNA was quantified using NanoDrop (ThermoFisher).

2.7.7 RNA processing for RNA seq by GenXPro

2.7.7.1 RNA integrity check, quantification and DNase I treatment

Upon arrival at GenXPro, the RNA was stored at -80°C until analysis by GenXPro, as described by Feil *et al.* (2017). The RIN was assessed again by Labchip GX II Bioanalyzer (Perkin Elmer), and DNA contamination was removed using Baseline-Zero DNase (Epicentre) following the GenXpro in-house protocol. The RNA was incubated in the presence of Baseline-Zero DNase and RiboLock RNase inhibitor (Thermo Fisher Scientific) for 30 min at 37°C. Two volumes of RNA Binding Buffer (Thermo Fisher Scientific) and absolute ethanol (Roth) were added to the sample and the mixture was transferred to a Zymo-Spin™ IC Column (Zymo Research). The column was centrifuged 12,000 *x g* for 30 s, and the bound RNA was washed twice with RNA Wash Buffer (Zymo Research) and eluted in nuclease-free water (Zymo Research) as per the manufacturer's protocol. RNA was re-quantified via the fluorescence-based Qubit™ RNA HS Assay (Thermo Fisher Scientific) following the manufacturer's protocol.

2.7.7.2 Ribosomal RNA depletion and mRNA enrichment

Total RNA was treated with Ribo-Zero rRNA removal kit (Illumina) to remove rRNA and enrich mRNA following the manufacturer's protocol. Briefly, the magnetic beads were washed twice with nuclease-free water and resuspended in Magnetic Bead Resuspension Solution and RiboGuard RNase Inhibitor. To hybridise the beads with the probes, RNase-free water, Ribo-Zero Reaction Buffer and Ribo-Zero Removal Solution were added to the beads and heated to 68°C for 10 min. Five hundred ng total RNA was added to the mixture and incubated at room temperature for 5 min then 50°C for

5 min. The tube was placed on a magnetic stand and the depleted RNA was removed in the supernatant and transferred to a separate tube. The enriched mRNA was purified using a Zymo-Spin Column (Zymo Research), and the eluted mRNA was checked for rRNA contamination by Labchip GX II Bioanalyzer.

2.7.7.3 Preparation of cDNA fragment libraries and RNA seq.

The NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina (Illumina) was used to prepare the cDNA fragment libraries. The enriched mRNA was incubated for 15 min at 94°C to fragment into pieces ~200 nt. NEBNext Strand Specificity Reagent and NEBNext First Strand Synthesis Enzyme Mix were added to the reaction. To enable reverse transcription for first-strand cDNA synthesis, the mixture was heated for 10 min at 25°C, 15 min at 42°C and 15 min at 70°C, before cooling to 4°C. NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix (10X), NEBNext Second Strand Synthesis Enzyme Mix and nuclease-free water were added to the mixture, and the mixture was heated for 1 h at 16°C to enable second-strand cDNA synthesis. The ds cDNA was purified using NucleoMag® NGS Clean-up and Size Select (Machery-Nagel). Briefly, the sample was mixed with NucleoMag® NGS beads in a 1:1 ratio, incubated at room temperature for 5 min, and then the beads were separted from the supernatant using a NucleoMag® SEP magnetic separator for 5 min. The supernatant was removed and discarded. Beads were washed twice with 80% ethanol, and dried by incubating at room temperature for 5 – 15 min. End repair was performed on the ds cDNA library followed by ligation of adaptors and the purified DNA fragment library was eluted in elution buffer. To determine appropriate cycle numbers for selective enrichment of library fragments by high fidelity PCR, qRT-PCR (Applied Biosystems) was performed using KAPA Hifi polymerase (Roche) with EvaGreen® (Biotium). NEBNext Multiplex

Oligos for Illumina (Dual Index Primers) and KAPA Hifi polymerase was used for selective enrichment by high fidelity PCR. PCR products were purified twice using NucleoMag SPRI beads and the quality of the final library was assessed on Labchip GX II Bioanalyzer.

2.7.7.4 Transcriptome Sequencing

Indexed and purified libraries were loaded together onto a flow cell, sequencing was carried out on the Illumina NextSeq 500 platform (paired-end, 2 X 75 bp per read).

2.7.7.5 RNA-Seq alignment, coverage and differential expression analysis

Sequencing quality was assessed using FastQC, and Illumina adapter sequences, and low-quality base pairs were removed using CutAdapt version 1.9 (Martin 2011). Reads were mapped to the complete sequenced genome of reference strains EGDe (ENSEMBL ASM19603v1) using Bowtie 2 v 2.2.4 with standard parameters and sensitive-local (Langmead and Salzberg 2012). BAM alignment files were used as input for read counting using htseq-count (HTSeq version 0.6.0) (Anders *et al.* 2015). Differential expression analyses were performed using DESeq2 in R v 3.2.2 (Love *et al.* 2014). The differential expression was reported as $\log_2$ fold changes, wtih p-values adjusted by the DESeq2 default Benjamini-Hochberg (BH) adjustment method and genes with a >2-fold change in expression and a p-value < 0.05 were considered as DE.

2.8 Growth assays

2.8.1 Growth curves

Five microliters of an overnight culture in BHI grown at the appropriate temperature were diluted in 5 mL culture medium (1:1000). Three hundred microliter aliquots were pipetted into a 100-well honeycomb plate (ThermoFisher), with three technical replicates per experiment and three blank controls for each culture medium. The plate was incubated for 48 h in a Bioscreen C at the appropriate temperature with continuous shaking, and OD_{600} was measured hourly. The values were exported to an Excel spreadsheet and growth curves were generated from the OD_{600} values normalised by subtracting the average of the three blank controls for the particular culture medium. The specific growth rate (μ) was calculated using the equation:

$$log_{10}N2 - log_{10}N1 = \frac{\mu(t2 - t1)}{2.303}$$

N refers to the cell concentration and *t* refers to time.

2.8.2 Biofilm assay

This assay was adapted from Feehily (2014) and O'Donoghue (2017). One millilitre aliquots of an overnight culture were taken, centrifuged at 13,000 *x g* for 2 min in a microcentrifuge (Eppendorf). The supernatant was discarded and the cells were washed once in 1 mL PBS, and resuspended in 1 mL PBS. Five microliters of the cells were inoculated into 5 mL (1:1000 dilution) of appropriate culture medium. Two hundred microliter aliquots were pipetted into a flat-bottomed 96-well plate, with 6 technical replicates per culture. Six wells were filled with sterile media to provide a blank, and

all empty wells were filled with sterile PBS to reduce evaporation. The plate was incubated for 48 h at an appropriate temperature without shaking.

After incubation, the media was removed from the wells by pipetting, with care being taken to minimise disturbance of the biofilm. The wells were washed 3 times with 200 µl PBS, and dried at 45°C for 45 min. One hundred and fifty microliters of 1% (w/v) crystal violet solution was added to each well, and the plates were incubated at 37°C for 30 min. The crystal violet was removed, and the plate was washed twice with 200 µl PBS. To ensure complete removal of any unabsorbed PBS, the plate was washed a third time under running deionised water and tapped upside down on paper towel to ensure optimal removal of the liquid. One hundred and fifty microliters of 95% ethanol were added to each well, and the plate was incubated at room temperature with gentle shaking to allow the crystal violet to dissolve. The Tecan Sunrise plate reader was used to measure the OD_{595} and the values were exported onto an Excel spreadsheet. Biofilm formation was calculated for each strain by subtracting the average OD_{595} value for the 6 wells filled with sterile media from the OD_{595} of each of the sample wells. A Dunn's multiple comparisons test was used to determine the statistical significance of each of the deletion mutants compared to the wild-type. P values of ≤ 0.05 were considered to be significant.

2.8.3 Quantitative fluorescence spectroscopy

Ten microliters of an overnight culture were diluted in 1 mL media (1:100) in the well of a 48-well plate (CellStar). The OD_{600} and the fluorescence (485 excitation/530 emission) were measured using a SpectraMax M2 plate reader. The plates were wrapped in aluminium foil and incubated at an appropriate temperature, shaking at 80 rpm. The OD_{600} and the

fluorescence (485 excitation/530 emission) were measured at 2 h intervals for 12 h and a final measurement was taken at 24 h.

To prepare the standard curve required for the data analysis step of the experiment, a 1.5 mL aliquot of an overnight culture was taken for each growth medium tested, and was centrifuged *13000 x g* for 2 min. The cells were washed once in PBS, and resuspended in 1.5 mL of the appropriate culture medium. A 1 mL aliquot was taken for each sample and pipetted into a 48-well plate. Each sample was serially diluted to 10^{-4} in culture medium, and the OD_{600} and the fluorescence (485 excitation/530 emission) were measured using a SpectraMax M2 plate reader.

To analyse the data, the OD_{600} reading of the blank culture well was subtracted from each experimental reading obtained during the experiment. A standard curve was generated by plotting the OD_{600} and fluorescence measurements obtained from the standard curve experiment. The background fluorescence value for each experimental OD_{600} measurement was interpolated from the standard curve, and subtracted from the experimental fluorescence reading obtained for each sample. The standard curve was required to determine the proportion of detected fluorescence occurring due to cell density and not the eGFP protein.

2.8.4 Growth curve in the presence of H_2O_2

An overnight culture grown in DM at the appropriate temperature was diluted to OD_{600} 0.05 in fresh DM. For each strain, a culture with and without 0.045% H_2O_2 was prepared to provide a negative control. The flasks were incubated, shaking at 70 rpm, in darkness at the appropriate temperature for 32 h, with OD_{600} readings taken at 2 h intervals. The experiment was

performed with biological triplicates, and the data was analysed using a two-way ANOVA with Dunnett's multiple comparisons test.

2.9 *Galleria mellonella* virulence assays

The established *Galleria mellonella* virulence model was selected to measure the virulence of the strains in this study. The *G. mellonella* larvae (SAS Sud Est Appats) were stored in darkness with wood shavings at room temperature. A 1 mL aliquot of overnight culture was taken and centrifuged at *5000 x g* for 10 min. The pellet was washed once in PBS and resuspended in PBS. The OD_{600} was measured and the culture diluted to OD_{600} 0.09 – 0.11 in PBS. A microneedle (Hamilton) was used to inject 10 µl of culture into the second last pseudopod of the larvae. Ten technical replicates and three biological replicates were performed. For each experiment, 10 control larvae were injected with 10 µl each sterile PBS. The larvae were incubated at 37°C for 7 days, and percentage survival was recorded at 24 h intervals. Dead larvae were removed at each inspection time. The data were analysed using a two-way ANOVA with Dunnett's test to correct for multiple comparisons.

2.10 Fluorescence microscopy

The overnight culture of the eGFP reporter strain was diluted in NB with supplements to an OD_{600} 0.01, and incubated in flasks shaking (160 rpm) at 30°C, in darkness. Samples for microscopy were taken after 24 hours of incubation, and prepared as described before (Utratna *et al.,* 2012). Briefly, 1 mL aliquots of the culture were centrifuged and washed twice in equal volume of PBS. Subsequently the pellet was resuspended in PBS to an OD_{600}

1, and observed under the microscope (Olympus BX41), 100X. For each sample, phase contrast and the eGFP fluorescence (ex/em 480/530) images were taken with the CellB software and overlaid using the ImageJ software. Representative images of each slide were taken. The experiment was performed with 2 biological replicates.

Chapter 3: Growth phase, temperature and ethanol exposure influence the resistance of *Listeria monocytogenes* to photodynamic inactivation

3.1 Abstract

Listeria monocytogenes is an important food-borne pathogen found in ready-to-eat products with tolerance to many stress treatments commonly utilised during food preservation. In this study the influence of environmental conditions and stresses on the sensitivity of *L. monocytogenes* to blue light was investigated. Photodynamic inactivation assays were used to measure the resistance of *L. monocytogenes* to blue light under various growth conditions. Growth phase and temperature were found to have a marked impact on light sensitivity, with stationary phase cells displaying significantly increased resistance compared to exponentially growing cells, and cells grown at 30˚C showing a marked increase in resistance compared to cells cultured at 37˚C. The role of the stress-inducible sigma factor σ^B in light resistance was confirmed at 30˚C but not at 37˚C. The transcription of three σ^B-dependent genes (*sigB*, *opuCA*, and *lmo2230*) in response to blue light was measured, with evidence of σ^B activation occurs at 30˚C but not at 37˚C. This effect is unlikely to be due to temperature effects on expression of the blue light sensor Lmo0799, since protein levels were similar at both temperatures. Finally, pre-exposure of *L. monocytogenes* to ethanol induced significant protective effects against blue light, independent of both σ^B and the ethanol-sensing two-component systems LisRK and CesRK. Overall the study identifies environmental conditions impacting light sensitivity in *L. monocytogenes* and shows that the σ^B activation pathway is essentially blind to light at physiological temperatures.

3.2 Introduction

The Gram positive rod *Listeria monocytogenes* is a ubiquitous organism in the environment and can cause infection when it enters the host via the food chain. Foods which are commonly considered to be at a greater risk of contamination by *L. monocytogenes* include ready-to-eat food products e.g. deli meats and smoked salmon, unpasteurised milk and dairy products e.g. soft cheeses, and raw sprouts (NicAogáin and O'Byrne 2016). The ability of *L. monocytogenes* to persist within ready-to-eat foods has been attributed to its resistance to food preservation regimes such as high salt concentrations, acid and alkali stress, and cold stress (Lou and Yousef 1997, Koutsoumanis *et al.* 2003, Chan *et al.* 2007, Bergholz *et al.* 2012, Ivy *et al.* 2012). Photodynamic inactivation (PDI) of *L. monocytogenes* by blue light has been shown to be an effective bactericidal mechanism (Maclean *et al.* 2009, Endarko *et al.* 2012), which could potentially be utilised in food processing environments as an adjunct to existing food preservation measures. However, the factors that influence sensitivity to blue light are not well understood and thus this study sought to investigate how the culture conditions and the presence of environmental stressors might influence susceptibility to PDI.

The resistance of *L. monocytogenes* to environmental stresses has been partly attributed to the alternative sigma factor SigB (Ferreira *et al.* 2003, Chaturongakul and Boor 2006, Utratna *et al.* 2011). The stressosome is a 1.8 megadalton protein complex that acts a signal integration hub, enabling the activation of the SigB signalling cascade in response to environmental stress (Marles-Wright *et al.* 2008). The stressosome complex is composed of the RsbT, RsbS and RsbR proteins (Hecker *et al.* 2007), as well as the four RsbR paralogues Lmo0161, Lmo0799, Lmo1642 and Lmo1842 (Impens *et al.* 2017). Of these paralogues, Lmo0799 has been characterised as a blue light

sensor, whose deletion alters some SigB–dependent phenotypes in the presence of blue light, such as motility and colony morphology (Ondrusch and Kreft 2011, Tiensuu *et al.* 2013). Amino acid sequence analysis of Lmo0799 reveals a high level of homology with the blue light sensor protein, YtvA, of *Bacillus subtilis*, including a cysteine residue at position 56 required for Flavin mononucleotide (FMN) adduct formation upon blue light exposure (Ondrusch and Kreft 2011). The FMN adduct is required to activate the kinase activity of RsbT in response to blue light, ultimately leading to the activation of the SigB signalling cascade. Although σ^B is required for resistance to blue light, somewhat paradoxically, neither the deletion of *lmo0799* from the genome, nor the conversion of Cys56 to an alanine residue, generates a light-sensitive phenotype in *L. monocytogenes,* although it does result in altered light-associated phenotypes such as colony ring formation and altered motility (O'Donoghue *et al.* 2016).

Certain stresses that *L. monocytogenes* is exposed to during food preservation, such as osmotic and acid stress, are also encountered within the host, and the effect of temperature on the ability of *L. monocytogenes* to adapt to these stresses has been studied (Bergholz *et al.* 2012, Shen *et al.* 2014). When changes in gene transcription in response to osmotic stress at both 7°C and 37°C were measured, 888 genes showed altered transcriptional profiles between the two temperatures (Bergholz *et al.* 2012). This set of genes included *sigB* and several other SigB-dependent genes involved in adaptation to stress conditions and pathogenesis (Bergholz *et al.* 2012). Similarly, it has been demonstrated that, while *L. monocytogenes* can display an adaptive response to acid stress at 30°C (Davis *et al.* 1996), cells are unable to show the same adaptation at 4°C (Shen *et al.* 2014).

It has been known for over 30 years that *L. monocytogenes* only expresses flagella at temperatures below 37°C (Peel *et al.* 1988), however a Δ*sigB* deletion mutant has increased motility at 37°C compared to the wild-type (Raengpradub *et al.* 2008). The transcriptomic study by Toledo-Arana *et al.* (Toledo-Arana *et al.* 2009) identified a SigB promoter upstream of *mogR,* a transcriptional repressor, that enables the transcription of three genes involved in flagellar biosynthesis, *lmo0675, fliP* and *fliQ.* Although this transcript is over-expressed in stationary phase, transcription is unaffected by temperature (Toledo-Arana *et al.* 2009). Therefore, growth temperature is an important variable, whose influence on the susceptibility of *L. monocytogenes* to PDI should be considered.

In the present study we also sought to investigate the influence of environmental stressors that could be present in a food processing environment on light tolerance. Acid and salt are important food preservation factors that *L. monocytogenes* demonstrates a SigB-dependent response to (Ferreira *et al.* 2001, Utratna *et al.* 2011), while ethanol is an important antimicrobial used in the food processing industry that has been shown to interact with other antimicrobial agents, altering their effectiveness against *L. monocytogenes* (Oh and Marshall 1993, Brewer *et al.* 2002). Pre-exposure of *L. monocytogenes* to sub-lethal doses of any of these stresses has been shown to induce resistance to lethal hydrogen peroxide stress (Lou and Yousef 1997). The LisRK and CesRK two-component regulatory systems of *L. monocytogenes* are involved in ethanol tolerance (Kallipolitis *et al.* 2003, Nielsen *et al.* 2012), with the Δ*lisK* mutant showing an enhanced growth phenotype in the presence of sub-lethal ethanol (Cotter *et al.* 1999).

3.3 Results

3.3.1 Growth temperature and phase influence blue light resistance in *L. monocytogenes*

In the present study, we examined the influence of growth phase on the resistance of *L. monocytogenes* to killing by blue light. EGDe wild-type cells were grown to either stationary or early exponential phase (OD ~0.2) at 37°C, and exposed to 35 mW cm^2 470 nm (blue) light over an 8h period. After 4 h and 6 h exposure to blue light, there was significantly ($p = \leq 0.001$) more killing of cells in the exponential phase of growth compared to those in stationary phase (Fig. 3.1A). To determine the role of SigB in the effect of growth phase on tolerance, the survival of the wild-type and Δ*sigB* mutant strains at stationary and exponential phase were compared after 6 h of exposure to 35 mW cm^2 470 nm (blue) light at 30°C. After 6 h, the wild-type exponential phase cells showed significantly ($p = \leq 0.001$) less survivors compared to the wild-type stationary phase cells. In contrast, the Δ*sigB* mutant showed no significant ($p = >0.05$) differences in the number of survivors between the two growth phases (Fig. 3.1B). Finally, we examined the effect of growth temperature on the resistance of *L. monocytogenes* to blue light. Cells were grown to exponential phase (OD ~0.2) at 30°C and 37°C, and exposed to 35 mW cm^2 470 nm light. After 6 h, the wild-type cells grown at 30°C had a significantly ($p = \leq 0.01$) higher number of survivors, in excess of two orders of magnitude, compared to those grown at 37°C (Fig. 3.1C). At 30°C, the Δ*sigB* mutant had a significantly ($p = \leq 0.05$) reduced percent survival compared to the wild-type. However, unexpectedly at 37°C the Δ*sigB* mutant had a significantly ($p = \leq 0.01$) greater survival than the wild-type (Fig. 3.1C). These results suggest that both growth phase and growth temperature influence the resistance of *L. monocytogenes* to blue light, with stationary phase cells and cells grown at 30°C showing less

sensitivity. The results also suggest that the role of σ^B in light resistance may vary as a function of growth phase and temperature.

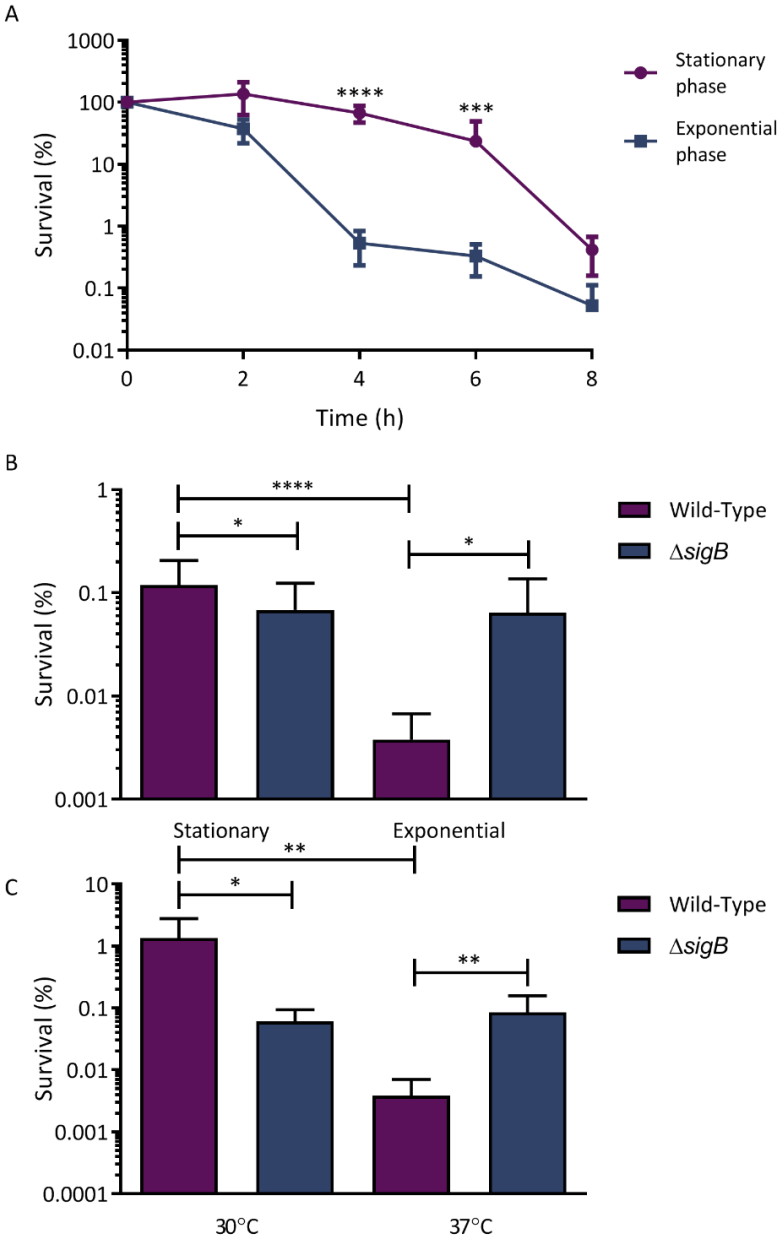

Figure 3. 1. Sensitivity of *L. monocytogenes* to visible light is affected by growth phase and temperature.

(A and B) Cultures were grown to exponential and stationary phase at 37°C, centrifuged, washed once in PBS and resuspended in PBS to OD_{600} 1, then exposed to 35 mW cm^{-2} 470 nm light for (A) 8 h or (B) 6 h. (C) Cultures were grown to exponential phase at 30°C or 37°C centrifuged, washed once in PBS and resuspended in PBS to OD_{600} 1, then exposed to 35 mW cm^{-2} 470 nm light for 6 h. Samples were taken at 0 h and the reported time points and cfu/mL were calculated. Error bars represent SD from three technical replicates, plated in triplicate, of two independent replicates. Statistical significance was determined using either two-way ANOVA with Dunnett's multiple comparisons test (A) or one-way ANOVA with Sidak's multiple comparisons test (B and C). (*: $p = \leq 0.05$; **: $p = \leq 0.01$; ***: $p = \leq 0.001$; ****: $p = \leq 0.0001$).

3.3.2 Activation of SigB by blue light is temperature dependent

As we had shown that the contribution of σ^B to surviving blue light stress in *L. monocytogenes* is dependent on the growth temperature, the influence of growth temperature on σ^B-dependent gene transcription in the presence of sub-lethal blue light (0.6 mW cm^{-2}) was investigated. The transcriptional response of *sigB,* and two other σ^B-dependent genes, *lmo2230* and *opuCA* ,was measured following exposure to blue light at 30˚C and 37˚C over a period of 30 min. Cells cultured at 30°C showed a significant (p = ≤0.05) increase in the transcription of all three genes in the presence of blue light compared to the dark control over a 30 min period (Fig. 3.2). However, in cells cultured at 37°C there were no significant differences in the levels of transcription of either *sigB* or *lmo2230* in the presence or absence of blue light. A small but significant (p = <0.0001) difference was seen in the transcription level of *opuCA* after 10 min, but this difference was not seen after 20 min or 30 min (Fig. 3.2C). Taken together the data suggest that the activation of σ^B by blue light is temperature-dependent, with a greater increase in activity demonstrated at 30°C than at 37°C, a result that may help to explain the differences in sensitivity to light at these temperatures (Fig. 3.2)

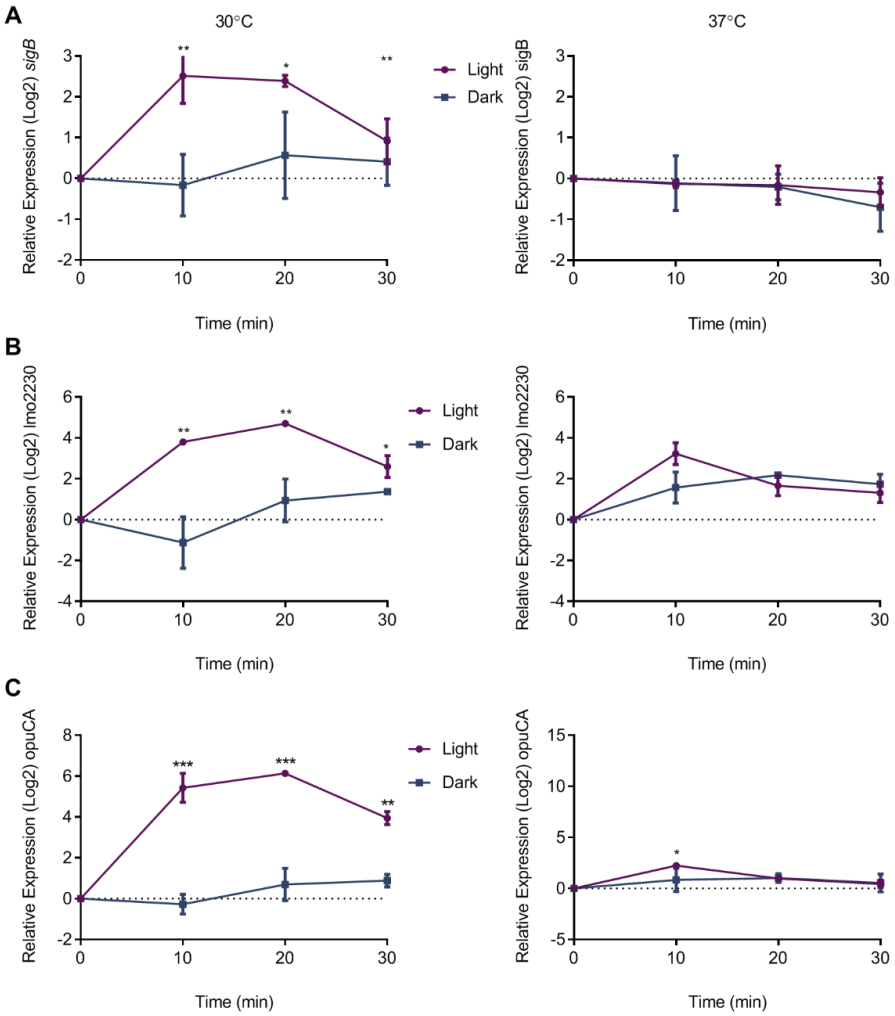

Figure 3. 2. Exposure to visible light significantly increases the transcription of *sigB*, *lmo2230* and *opuCA* compared to the dark control at 30°C but not 37°C.

Cells were grown to OD ~0.2 at 30°C or 37°C and exposed to 0.6 mW cm-2 470 nm light for 30 min at the same temperature, with RNA samples collected at 10 min intervals. Relative transcription of *sigB* (A), *lmo2230* (B) and *opuCA* (C) was measured by RT-PCR. Error bars represent SD from two independent replicates. Statistical significance was determined by two-way ANOVA with Dunnett's multiple comparisons test. (*: $p = \leq 0.05$; **: $p = \leq 0.01$; ***: $p = \leq 0.001$; ****: $p = \leq 0.0001$).

3.3.3 The expression of *lmo0799* is not temperature dependent

One possible explanation for the apparent lack of light-mediated σ^B activation at 37˚C was that the light sensor protein Lmo0799 might not be

expressed at this temperature. To determine whether the reduced activation of σ^B by blue light at 37°C compared to 30°C was due to the reduced transcription of *lmo0799* at 37°C compared to 30°C, the difference in *lmo0799* transcription at 30°C compared to 37°C was measured by RT-PCR over a 30 min period. No significant (p = >0.05) differences were detected in the transcription of *lmo0799* in cells cultured at 30°C or 37°C (Fig. 3.3A). In addition, we measured the changes in *lmo0799* transcription in the presence and absence of blue light in cells cultured at 30°C and 37°C. Again, no significant (p = >0.05) differences were detected (Fig. 3.3A). To confirm that the levels of the Lmo0799 sensor protein is not affected by growth temperature, we quantified the levels of Lmo0799 via Western blotting using anti-Lmo0799 antibodies. The levels of Lmo0799 were found to be unaffected by growth temperature (Fig. 3.3B). Together these results suggest that *lmo0799* is not affected by either growth temperature or blue light at the transcriptional or translational levels.

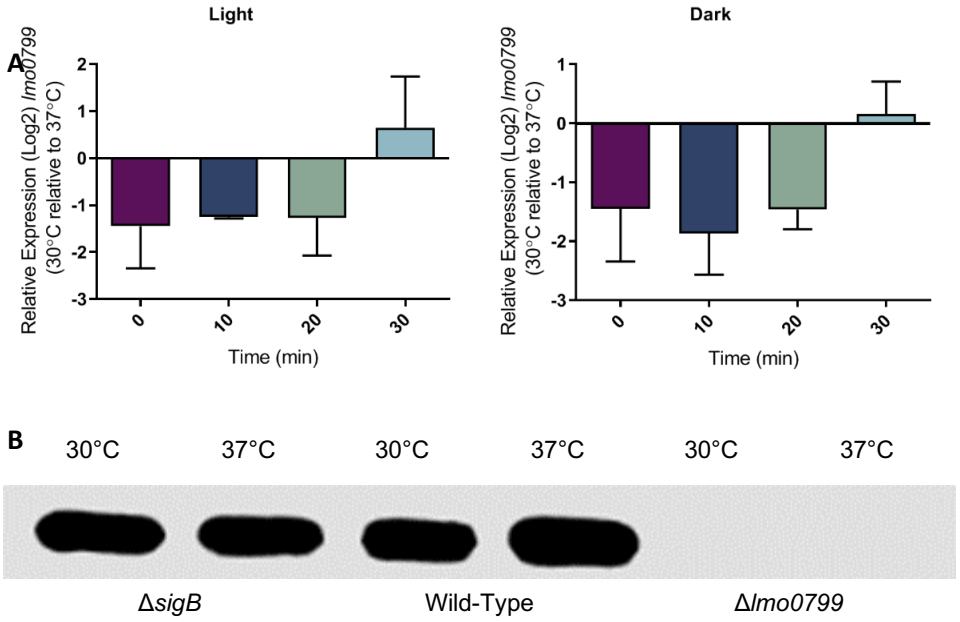

Figure 3. 3. The expression of lmo0799 is unaffected by temperature.

(A) Cells were grown to OD ~0.2 at 30°C or 37°C and exposed to 0.6 mW cm-2 470 nm light for 30 min at the same temperature, with RNA samples collected at 10 min intervals. Relative transcription of *lmo0799* to T0 at each temperature was measured by RT-PCR. Error bars represent SD from two independent replicates. Statistical significance was determined by two-way ANOVA with Dunnett's multiple comparisons test. (B) Protein extracts from stationary phase cultures of the wild-type, Δ*sigB* and Δ*lmo0799* strains grown at 30°C or 37°C in darkness were standardised to 0.55 mg mL^{-1} and separated via SDS-PAGE. The levels of Lmo0799 in cultures was determined by Western blot with polyclonal anti-Lmo0799 antibodies, with the Δ*lmo0799* strain as a negative control. Image is representative of three independent replicates.

3.3.4 Ethanol or salt pre- exposure provides cross-resistance of *L. monocytogenes* to blue light

To determine whether acid, salt or ethanol could also provide cross-protection against blue light, the resistance of cells with and without pre-exposure to these stresses was investigated. Cells were grown to exponential phase (OD ~0.2) at 30°C, incubated in BHI broth alone or BHI broth either acidified to pH 5.8 or supplemented with either 0.5 M NaCl or4% ethanol for 1 h, and exposed to 35 mW cm^2 470 nm light. After 5 h,

both the wild-type and Δ*sigB* mutant cultures pre-exposed to either 0.5 M NaCl or 4% ethanol had significantly (p = ≤0.05) more survivors after exposure to blue light compared to cells not pre-exposed to ethanol (Fig. 3.4A). Cells that were pre-exposed to pH 5.8 did not show a significant (p = >0.05) increase in resistance to blue light. To test whether this effect was independent of growth temperature, the experiment was repeated with cultures grown at 37°C. After 5 h exposure, the cells pre-exposed to 4% ethanol had significantly greater survival (p = ≤0.0001) compared to those not pre-exposed to ethanol, independent of SigB (Fig. 3.4B). At 37°C, pre-exposure to 0.5 M NaCl induced a significantly (p = ≤0.05) protective response in the wild-type but not in the Δ*sigB* mutant (p = >0.05), and pre-exposure to pH 5.8 did not result in a significant (p = >0.05) increase in tolerance. These results suggest that pre-exposure to ethanol can provide *L. monocytogenes* with cross-resistance to blue light.

To investigate whether either the LisRK or CesRK two-component systems was involved in the cross-protection provided by ethanol to blue light, Δ*lisR* and Δ*cesR* and *ΔcesK mutants* were used. As the mutants were only available in the LO28 background, the LO28 wild-type strain was used as the negative control for these strains. The mutants and their isogenic parental strain were grown to exponential phase (OD ~0.2) at 30°C, incubated in BHI broth with or without 4% ethanol for 1 h, and exposed to 35 mW cm^2 470 nm light. After 5 h, the wild-type, Δ*cesR* and Δ*cesK* strains showed a significant (p = ≤0.01) increase in resistance to killing by blue light after pre-exposure to 4% ethanol (Fig. 3.5), indicating that CesRK is not responsible for the induction of cross-protection against blue light by ethanol. Furthermore, the ethanol –induced light resistance phenotype was observed in both EGDe and LO28 (Fig. 3.5), indicating that this phenomenon is not a strain-specific artefact. The Δ*lisR* mutant did not show a significant (p = >0.05) increase in resistance to blue light after pre-exposure to 4% ethanol.

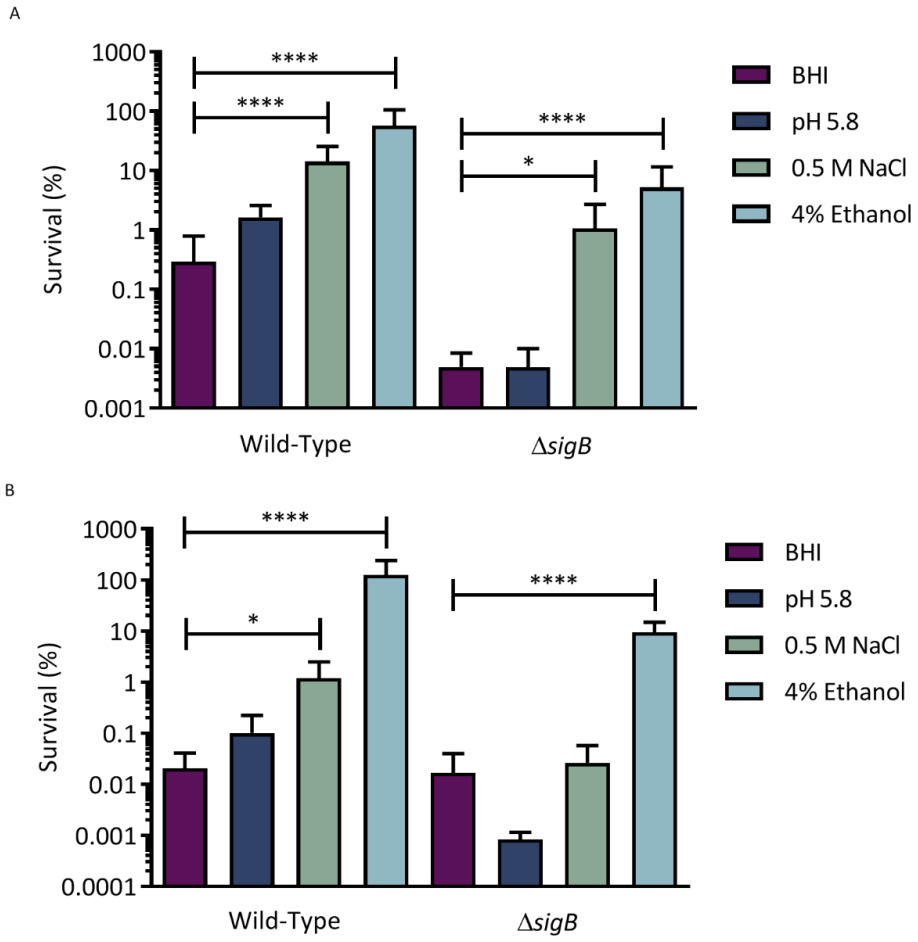

Figure 3. 4. Pre-exposure of *L. monocytogenes* to ethanol or salt induces resistance to blue light.

Cultures were grown to early exponential phase at 30°C (A) or 37°C (B) in BHI broth. Cells were centrifuged and resuspended in either BHI broth or BHI broth supplemented to either pH 5.8, 0.5 M NaCl or 4% ethanol. Cells were incubated at growth temperature for 1 h. Cells were centrifuged, washed once in PBS and resuspended in PBS, then exposed to 35 mW cm^{-2} 470 nm light for 5 h. Error bars represent SD from three technical replicates, plated in triplicate, of two independent replicates. Statistical significance between the BHI control and the mild stress conditions within each strain was determined using a Kruskal-Wallis test with Dunn's multiple comparisons test (*: $p = \leq 0.05$; ****: $p = \leq 0.0001$).

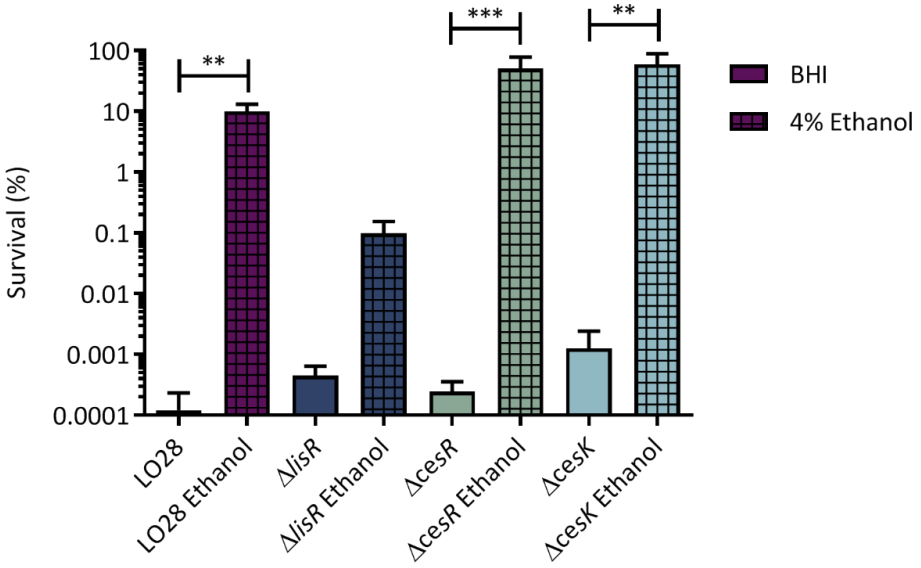

Figure 3. 5. LisR may be required for the induced resistance to visible light by pre-exposure to ethanol.

Cultures were grown to early exponential phase at 30°C in BHI broth. Cells were centrifuged and resuspended in either BHI broth or BHI broth supplemented with 4% ethanol. Cells were incubated at 30°C for 1 h. Cells were centrifuged, washed once in PBS and resuspended in PBS, then exposed to 35 mW cm^{-2} 470 nm light for 5 h. Error bars represent SD from three technical replicates, plated in triplicate, of two independent replicates. Statistical significance was determined using a Kruskal-Wallis test with Dunn's multiple comparisons test (*: $p = \leq 0.05$; ****: $p = \leq 0.0001$).

3.4 Discussion

3.4.1 Influence of growth phase and temperature on sensitivity to blue light.

Growth phase and temperature were shown here to alter the sensitivity of *L. monocytogenes* to blue light. In addition, the role of SigB in protecting against blue light was found to be temperature dependent. The influence of growth phase on the sensitivity of *L. monocytogenes* to environmental stresses has previously been reported for acid stress (Davis *et al.* 1996), heat (Lou and Yousef 1997) and hydrostatic pressure (Mackey *et al.* 1995,

Saucedo-Reyes *et al.* 2009), with cells in the stationary phase of growth to be more resistant to these stresses than those in the exponential phase. Utratna *et al.* (Utratna *et al.* 2011), demonstrated a proportional increase in SigB activation as growth phase increased in response to osmotic stress. As we have previously identified a role for SigB in blue light tolerance at 470 nm (O'Donoghue *et al.* 2016), we hypothesise that the increased resistance of *L. monocytogenes* to blue light in stationary phase is due to increased SigB activity in stationary phase. The data in this study suggest that this is the case, as the Δ*sigB* mutant showed no alteration in resistance to blue light in response to changes in growth phase (Fig. 3.1B).

The change in growth temperature from 30°C to 37°C significantly reduced the survival of the wild-type 100-fold, but the Δ*sigB* mutant was unaffected. While this result was unexpected, the presence of SigB has been associated with increased sensitivity of *L. monocytogenes* EGDe to hydrogen peroxide at 37˚C but not at 30˚C (Boura *et al.* 2016). As the mechanism by which blue light kills *L. monocytogenes* is thought to involve reactive oxygen species (O'Donoghue *et al.* 2016), it seems possible that the role of SigB could be similar during exposure to blue light or hydrogen peroxide. In agreement with our study, Boura *et al.* (2016) also demonstrated an increased sensitivity of the wild-type to hydrogen peroxide at 37°C compared to 30°C. Considering the deleterious effect of SigB at 37°C in resistance to blue light (Fig. 3.1B), we hypothesised that there may be a difference in the activity of SigB between the two temperatures in response to blue light exposure. This differential activation of σ^B by blue light at 30˚C and 37˚C was not due to a temperature-dependent change in the expression of *lmo0799*, since both the transcription and translation of *lmo0799* were unaffected by the growth temperature (Fig. 3.3). One possibility is that the FMN cofactor required for blue light sensing might associate with the sensor protein Lmo0799 in a temperature-dependent manner. In this regard it is noteworthy that Chan

et al. (Chan *et al.* 2013) demonstrated a reduced retention of the Lmo0799 FMN chromophore as temperatures increase above 26°C. Although somewhat unexpected, the finding that σ^B activation is absent at 37˚C does potentially make physiological sense. When *L. monocytogenes* is exposed to 37°C, it is most likely to be within a mammalian host, an environment where light exposure is essentially absent. There would be no selective pressure to retain the capacity to sense light at this temperature. As SigB is known to be active in the host, as *inlA* expression is under the control of SigB in the gastrointestinal tract (Toledo-Arana *et al.* 2009), it is possible that SigB is activated by the presence of acid and bile in this environment as opposed to temperature. At typical environmental temperatures outside the host (30˚C and below) the capacity to sense light and mount an appropriate protective response would be restored, thereby facilitating survival of any solar irradiance encountered.

3.4.2 Ethanol induce resistance to blue light, independently of SigB and CesRK

The second part of this study investigated the influence of alternative environmental stresses on the resistance of *L. monocytogenes* to blue light, a scenario that could arise in a food processing environment where acid and salt are routinely used for food preservation, and ethanol as a surface sanitiser. Here the presence of alcohol-based sanitisers could mitigate the effectiveness of a light based control measure. Previously, pre-exposure of *L. monocytogenes* to sub-lethal ethanol has induced cross-protection to hydrogen peroxide (Lou and Yousef 1997), a stress which is thought to also induce bacterial inactivation via reactive oxygen species, so it was unsurprising that ethanol induced high levels of cross-protection to blue light stress (Fig 3.4A). However, these results were in contrast to those obtained by McKenzie *et al.* (2014), who identified a role for sub-lethal

temperature, osmotic and acid stress in increasing the sensitivity of *L. monocytogenes* to blue light stress. It is important to note that the study by McKenzie *et al.* (2014) used light at 405 nm, as opposed to the 470 nm light used in the current study, as well as a reduced pH and increased osmolarity during the sub-lethal stress exposures, all of which may account to the different results obtained to those in the current study.

It has been shown that SigB is not required for the tolerance of *L. monocytogenes* to ethanol (Ferreira *et al.* 2001), although it has been shown to be a strong inducer of SigB activity in *B. subtilis* (Boylan *et al.* 1993). However, increased transcription of the SigB -dependent *opuCA* gene has been reported in response to ethanol exposure (Chaturongakul and Boor 2006). As the present study reported an increase in *opuCA* transcription in response to blue light exposure, the role of SigB in the cross-protective response between ethanol and blue light was investigated. However, the data suggest that the mechanism of ethanol-induced cross-protection to light is independent of SigB (Fig 3.4A and B).

Growth experiments in 5% ethanol identified potential roles for *resD, phoP, lmo1745, degU, cesR* and *lisR* in ethanol tolerance, maybe due to their ability to alter the cell envelope (Williams *et al.* 2005). In the current study, our use of Δ*lisR,* Δ*cesR* and Δ*cesK* mutants suggested that the CesRK two-component system, like SigB, is not involved in the cross-protective response between ethanol and blue light. While pre-exposure to ethanol did not induce a statistically significant level of resistance in the *ΔlisR* mutant, the 2-log increase in survival of this strain after pre-exposure to ethanol compared to the negative control should not be ignored. The deletion of LisR removes the response regulator, but not the sensor encoded by *lisK,* from the two-component system. Gene expression studies targeting genes known to be under the control of LisRK have shown that the deletion of *lisR* reduces, but

does not abolish the transcription of multiple genes whose expression has been shown to be regulated by LisRK (Nielsen *et al.* 2012). Therefore, it is possible that the deletion of *lisR* alone does not abolish the expression of the gene(s) mediating the ethanol-dependent resistance to blue light. The construction of a double Δ*lisR*Δ*lisK* mutant is required to fully elucidate the role of this two-component system in mediating the resistance of *L. monocytogenes* to blue light via ethanol exposure. Thus at present the mechanism involved in this protective effect remains unknown although two possibilities seem worth exploring. First there is some evidence that ethanol can exacerbate oxidative stress in *E. coli* (Belkin *et al.* 1996) and if this occurred in *L. monocytogenes* it could trigger a protective response to the oxidative stress induced by blue light (O'Donoghue *et al.* 2016). Secondly, ethanol is thought to produce a heat shock-like response in *Saccharomyces cerevisiae* (Piper *et al.* 1995) and in *E. coli* (Van Bogelen *et al.* 1987) and it is possible that such a response could confer cross protection against the damaging effects of light. Further work will be required to investigate these possibilities and identify the precise mechanism involved in the alcohol-induced protection against light.

3.5 Conclusions

In conclusion, this study has identified an aptitude for growth temperature in the tolerance of *L. monocytogenes* to blue light, and also for activating SigB in response to blue light. We have shown that these differences are unlikely to be due to temperature-dependent differences in *lmo0799* transcription or translation. In addition, we have shown an important capacity for ethanol in inducing cross-resistance to blue light independently of growth temperature, a finding that is of high importance to the food processing industry should PDI be developed as a control measure for *L. monocytogenes*.

Chapter 4: Whole transcriptome analysis of *L. monocytogenes* exposed to blue light offers novel insights into the role of SigB in blue light resistance

4.1 Abstract

Listeria monocytogenes is a food-borne pathogen that is ubiquitous in the environment, where it will encounter exposure to blue light. While previous work has demonstrated the ability of blue light to inactivate *L. monocytogenes,* and a role for SigB in resistance to this killing, little is known about the role of SigB or the blue light sensor protein, Lmo0799, in the response of *L. monocytogenes* to blue light. In the current study, we provide whole transcriptome analysis via RNAseq, offering novel insights into the changes in gene expression in response to blue light. The inclusion of the Δ*sigB* and *lmo0799* C56A, where a conserved cysteine residue that is hypothesised to be critical for blue light sensing in *L. monocytogenes* is mutated to an alanine residue, mutants in our analysis, enables us to elucidate the effects of these genes on whole genome transcription. The results of this study demonstrate that the majority of genes with altered expression in the presence of blue light are under the control of SigB. The partial activation of many of these genes in the absence of Cys56 suggests that SigB is activated in response to blue light via an alternative mechanism, possibly explaining why the absence of Cys56 does not induce sensitivity to blue light. Overall, this study offers novel insights into the role of SigB in resistance to blue light through the transcriptomic changes in *L. monocytogenes* in response to blue light exposure, both dependent and independent of SigB or Cys56.

4.2. Introduction

Listeria monocytogenes is an important food pathogen that is ubiquitous in the environment. In order to survive, the bacteria must be able to sense and respond to various environmental stimuli, including light. The exposure of

L. monocytogenes to solar irradiation in seawater results in a complete loss of viability within 80 h, demonstrating that light exposure in the environment, which would include UVA and UVB wavelengths as well as visible light, can be lethal to *L. monocytogenes* (NicAogáin *et al.* 2018). Several bacterial species possess Light, Oxygen or Voltage (LOV) domain proteins to sense and survive the presence of blue light, including the marine bacteria *Dinoroseobacter shibae* (Endres *et al.* 2015) and *Erythrobacter litoralis* (Zoltowski *et al.* 2013), and the pathogenic bacteria *Brucella abortus* (Sycz *et al.* 2015), *Listeria monocytogenes* (Ondrusch and Kreft 2011) and *Caulobacter cresentus* (Purcell *et al.* 2007). The LOV domain of *L. monocytogenes* was identified in a light-sensing protein known as Lmo0799, based on its sequence homology to YtvA, the blue light sensing protein of *Bacillus subtilis* (Ondrusch and Kreft 2011).

The stressosome protein complex was first discovered in *Bacillus subtilis* (Kim *et al.* 2004), and it is required to detect environmental stress signals and initiate the signalling cascade required to activate SigB (Marles-Wright *et al.* 2008). In *L. monocytogenes,* the stressosome is composed of a core of RsbS and RsbT proteins, with RsbR and its paralogue proteins embedded into this core (Impens *et al.* 2017). The blue light sensor Lmo0799 is an RsbR paralogue (Impens *et al.* 2017), however the sensory functions of the other four paralogues is currently unknown. As in *B. subtilis* (Gaidenko *et al.* 2006), the exposure of *L. monocytogenes* to blue light activates the alternative sigma factor SigB (Ondrusch & Kreft 2011), which is required to induce transcription of the general stress response regulon (O'Byrne and Karatzas 2008). The fortuitous discovery that oscillating cycles of light and dark results in a ringed colony morphology in *L. monocytogenes* further confirmed that Lmo0799 is required for SigB activation by light (Tiensuu *et al.* 2013). In the absence of either Lmo0799 or SigB, *L. monocytogenes* is

unable to form the ringed colony morphology in the oscillating cycles of light and dark (Tiensuu *et al.* 2013).

Several amino acids are conserved between YtvA and Lmo0799, including the cysteine residue at positions 62 and 56 in YtvA and Lmo0799, respectively, that is required for the formation of a photoadduct with the flavin mononucleotide cofactor in response to blue light (Gaidenko *et al.* 2006, O'Donoghue *et al.* 2016). In both the deletion mutant Δ*lmo0799* and the missense mutant Lmo0799 C56A, where this critical cysteine residue is mutated to an alanine, several phenotypes associated with the exposure of *L. monocytogenes* to blue light are abolished (O'Donoghue *et al.* 2016). These phenotypes include the inhibition of motility (Ondrusch and Kreft 2011), a ringed colony morphology in the presence of oscillating cycles of light and dark (Tiensuu *et al.* 2013), and inhibited growth in the presence of low doses of blue light (O'Donoghue *et al.* 2016). Therefore, the presence of this conserved cysteine residue is required to sense light in *L. monocytogenes,* and the mutant lacking it effectively behaves as a blind strain in respect to blue light.

Studies concerning several bacterial species, including *L. monocytogenes,* and their killing by light have been conducted. The sensitivity of *Staphylococcus aureus* to killing by blue light is highly dependent upon the environmental oxygen concentrations, with sensitivity increasing as oxygen levels increase (Maclean *et al.* 2008). A comparison between *Salmonella enteriditis, Escherichia coli* and *Campylobacter jejuni* demonstrated that while all three species could be killed by blue light, *C. jejuni* showed a significantly increased sensitivity to killing compared to the other two species (Murdoch *et al.* 2010). In a similar study comparing the sensitivity of *L. monocytogenes, S. enterica, S. sonnei* and *E. coli* to blue light, *L.*

monocytogenes was significantly more sensitive than the other species in liquid culture, but the results varied on solid surfaces (Murdoch *et al.* 2012). Taken together, the results of these studies suggest that blue light may have the potential to be an effective antimicrobial, however the response of bacteria to blue light needs to be understood to optimise the use of blue light as an antimicrobial.

While a role for the alternative sigma factor SigB in resistance to killing by blue light has been shown, somewhat surprisingly neither the Δ*lmo0799* nor the Lmo0799 C56A mutants show a change in sensitivity to killing by blue light (O'Donoghue *et al.* 2016). However, increased transcription of the SigB-dependent gene *lmo2230* in response to blue light requires Lmo0799 (Tiensuu *et al.* 2013), suggesting that *L. monocytogenes* may respond to blue light via an Lmo0799-independent mechanism. Despite evidence that blue light may be an effective antimicrobial, very little is known about the global transcriptomic response of *L. monocytogenes* to blue light, and indeed this is true of most non-phototrophic bacteria. The current study sought to elucidate the transcriptional response of *L. monocytogenes* to blue light via whole transcriptomic analysis in order to develop a deeper understanding of how this important food pathogen senses and responds to blue light.

4.3 Results

4.3.1 The blue light sensor is not required for blue light tolerance

In our previous work, we have shown that the absence of neither the blue light sensing protein, Lmo0799, nor the conserved cysteine residue within Lmo0799, Cys56, alters the resistance of *L. monocytogenes* to a low dose (8 mW/cm^2) of blue light, although at the same dose a mutant lacking *sigB*

displays a survival defect (O'Donoghue *et al.* 2016). In this study we sought to investigate whether Cys56 was required for resistance to much higher intensity blue light (35 mW/cm^2). EGDe wild-type, Δ*sigB* and *lmo0799* C56A (a mutant where residue Cys56 is changed to an alanine) cells were grown to

stationary phase at 30°C, and exposed to 35 mW cm^2 470 nm (blue) light. After 6 h, there was a 100-fold decrease in the survival of both the wild-type and C56A strains, with no significant (p = >0.05) difference between them (Fig. 4.1). There was a 10,000-fold decrease in Δ*sigB* mutant strain, which was significantly (p = ≤0.0001) lower than both the wild-type and C56A strains (Fig. 4.1). In agreement with our previous study, these results suggest that Lmo0799 Cys56 is not required for resistance to killing by blue light, but σ^B is required for blue light resistance under these conditions.

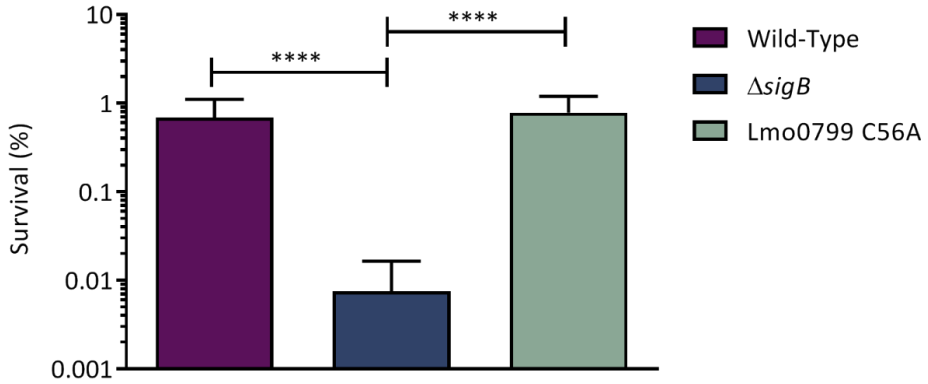

Figure 4. 1 Cys56 is not required for visible light tolerance.

Cultures were grown to stationary phase at 30°C in BHI broth. Cells were centrifuged, washed once in PBS and resuspended in PBS to OD_{600} 1, then exposed to 35 mW cm^{-2} 470 nm light for 8 h. Statistical significance was determined using a one-way ANOVA with Dunnett's multiple comparisons test. (*****: p = ≤ 0.0001).

4.3.2 The exposure of *L. monocytogenes* to blue light significantly alters the transcription of 603 genes.

To determine possible mechanisms that account for the increased sensitivity of the Δ*sigB* mutant to blue light compared to the wild-type and C56A strains, whole transcriptome analysis was carried out for all three strains in both the absence (dark) and presence of 0.6 mW cm^2 470 nm light. This low dose of light was chosen in line with previous work conducted by Ondrusch and Kreft (2011). In the presence of blue light, the wild-type strain the transcription of 603 genes was significantly (p = ≤0.05; log2 fold change ≥ 2) altered compared to the dark control (Fig. 4.2). Of these 603 genes, 308 were upregulated and 295 were downregulated, with *opuCC* showing the largest increase in transcription (6.47 log2 fold change) and lmo*0684* showing the greatest decrease in transcription (-4.63 log2 fold change). An analysis of the functional categories affected by blue light revealed that a large proportion of the downregulated genes are involved in cell envelope and cellular processes (Fig. 4.3A). When the downregulated genes involved in cell envelope and cellular processes were further divided into sub-categories, the mobility and chemotaxis sub-category was highly over-represented; 23.64% of significantly downregulated genes compared to 5.02% of the whole genome (Fig. 4.3B). Genes upregulated by blue light were distributed evenly across all functional categories. Taken together, these results indicate that exposure to low levels of blue light leads to significant changes in gene transcription, with a large proportion of the affected genes involved in mobility and chemotaxis.

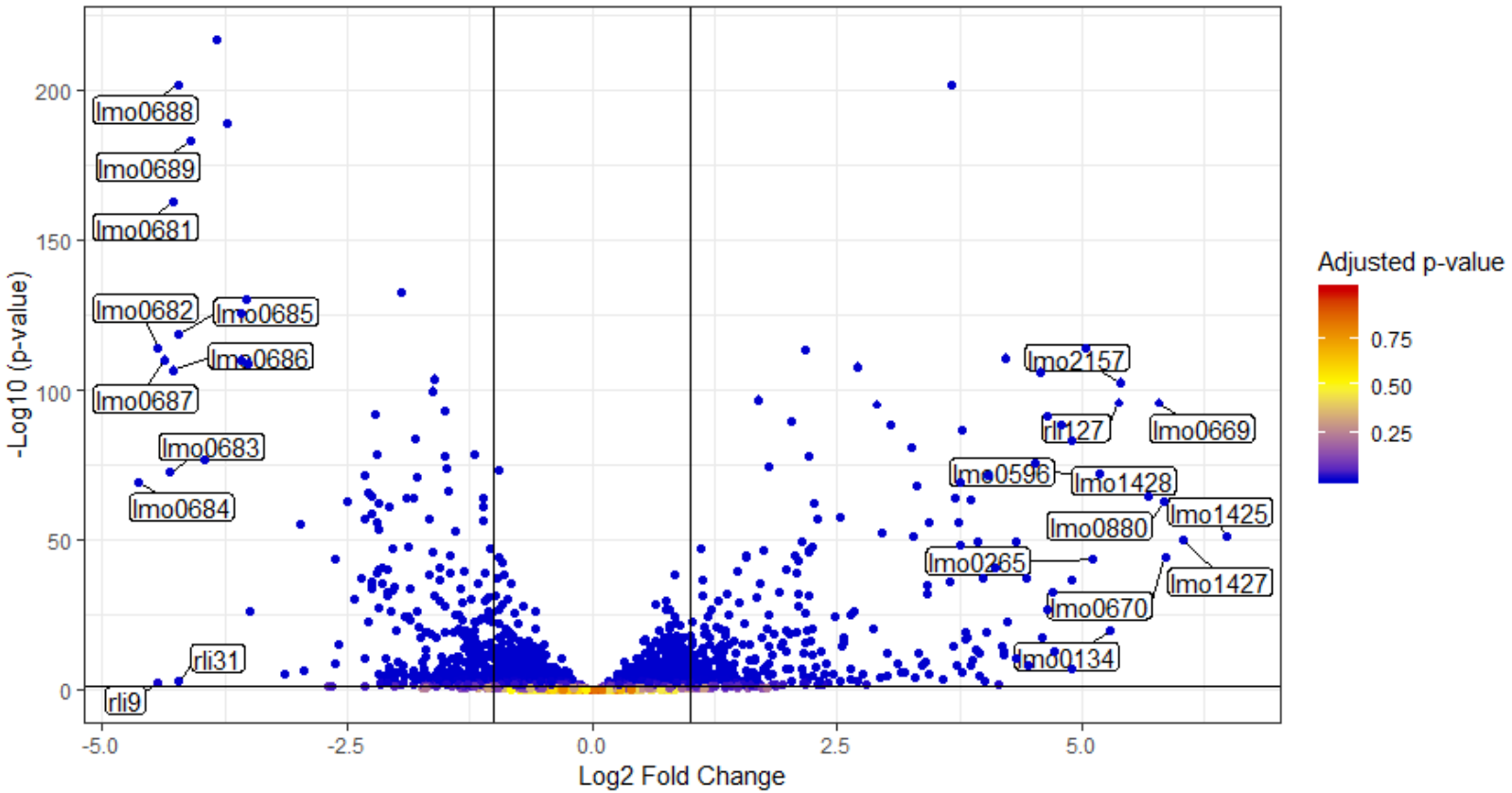

Figure 4. 2 The exposure of L. monocytogenes to visible light significantly alters the transcription of 603 genes.

RNA was sampled from exponentially growing cells exposed to either 0.6 mW cm^{-2} 470 nm light or darkness for 20 min at 30°C. Gene transcription was measured by RNA seq. and differential gene expression and p-values were determined using DESeq2.

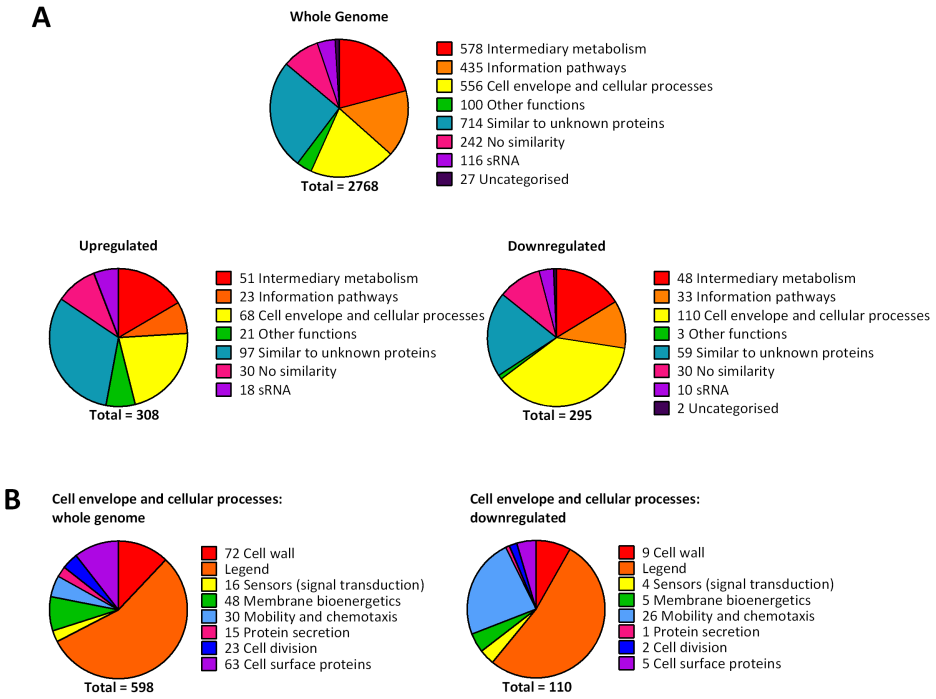

Figure 4. 3 Exposure to visible light significantly downregulates genes involved in cell envelope and cellular processes.

A) Genes significantly up or down regulated in response to visible light were divided into functional categories. The proportion of downregulated genes involved in cell envelope and cellular processes was greater than in the whole genome. B) Genes involved in cell envelope and cellular processes that were significantly downregulated in response to visible light were divided into sub-categories. The proportion of significantly downregulated genes involved in cell mobility and chemotaxis was greater than in the whole genome.

4.3.3 The transcriptional response to blue light is σ^B-dependent

To investigate the effects of the deletion of SigB on gene expression changes in response to blue light, whole transcriptome analysis was conducted using the Δ*sigB* mutant. In the absence of SigB, only 10 genes were found to be significantly altered in response to blue light exposure compared to the dark control (Fig. 4.4). In contrast to the wild-type, the Δ*sigB* mutant significantly increased the transcription of *rli78* and *lmo0544,* and significantly decreased the transcription of *lmo0481* and *lmo2818.* These genes were distributed across functional categories (Table 3). Overall, these results suggest that the

transcriptional response to blue light in *L. monocytogenes* is largely dependent on the stress inducible sigma factor σ^B, potentially accounting for the light-sensitive phenotype observed in the mutant lacking this sigma factor (Fig. 4.1).

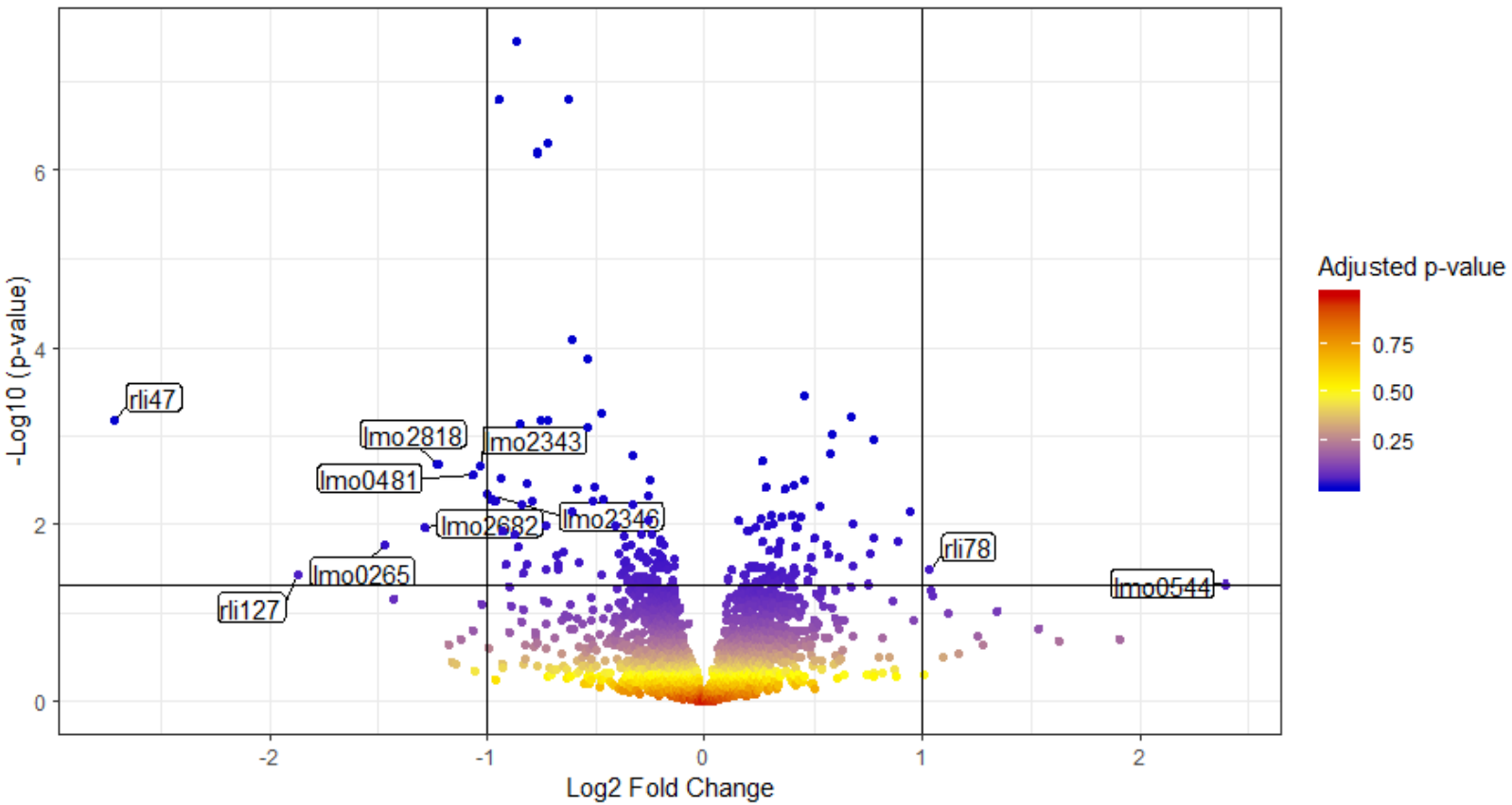

Figure 4. 4 In the absence of SigB, the transcription of only 10 genes is significantly affected by visible light.

RNA was sampled from exponentially growing cells exposed to either 0.6 mW cm^{-2} 470 nm light or darkness for 20 min at 30°C. Gene transcription was measured by RNA seq. and differential gene expression and p-values were determined using DESeq2.

Table 4. 1. Genes with significantly altered gene expression in the presence of blue light in a Δ*sigB* mutant.

Gene Name	Log2 Fold Change	Functional Category	RAST_Product
lmo0544	2.39	Transport/binding proteins and lipoproteins	PTS system, glucitol/sorbitol-specific IIC component
RatA-1 (rli78)	1.03	sRNA	Unknown
lmo2346	-1.00	From other organisms	ThiJ/PfpI family protein
lmo2343	-1.04	Detoxification	Coenzyme F420-dependent N5,N10-methylene tetrahydromethanopterin reductase and related flavin-dependent oxidoreductases
lmo0481	-1.07	From other organisms	Putative antigen
lmo2818	-1.23	Transport/binding proteins and lipoproteins	Putative transporter
kdpA	-1.28	Transport/binding proteins and lipoproteins	Potassium-transporting ATPase A chain
lmo0265	-1.47	Metabolism of amino acids and related molecules	Acetylornithine deacetylase
rli127	-1.87	sRNA	Unknown
sbrE (rli47)	-2.71	sRNA	Unknown

4.3.4 The absence of Cys56 reduces the number of genes with significantly altered expression in the presence of blue light to 77

In order to investigate possible mechanisms for the increased resistance of the *lmo0799* C56A mutant to blue light compared to the Δ*sigB* mutant, whole transcriptome analysis was carried out on the *lmo0799* C56A mutant in response to blue light. In this strain, which is unable to sense blue light

(O'Donoghue *et al.*, 2016), the transcription of 77 genes was altered compared to the dark control (Fig. 4.5). Of the genes with significantly altered transcription, 32 also showed significantly altered transcription in the wild-type, while 45 were uniquely affected in the C56A mutant strain. Of the genes whose transcription changed significantly in response to blue light exposure, there is an over representation of genes involved in intermediary metabolism and information pathways, and an under representation of genes involved in cell envelope and cellular processes, other functions, similar to unknown proteins, and sRNAs. Overall, these data suggest that *L. monocytogenes* is able to significantly alter the transcription of a larger number of genes in the absence of a functional light sensor (Lmo0799) than in the absence of SigB, which suggests that σ^B activation may still be occurring in this strain independently of the ability to sense light directly. If this is the case it may help to explain the absence of a light-sensitive phenotype in the C56A mutant strain.

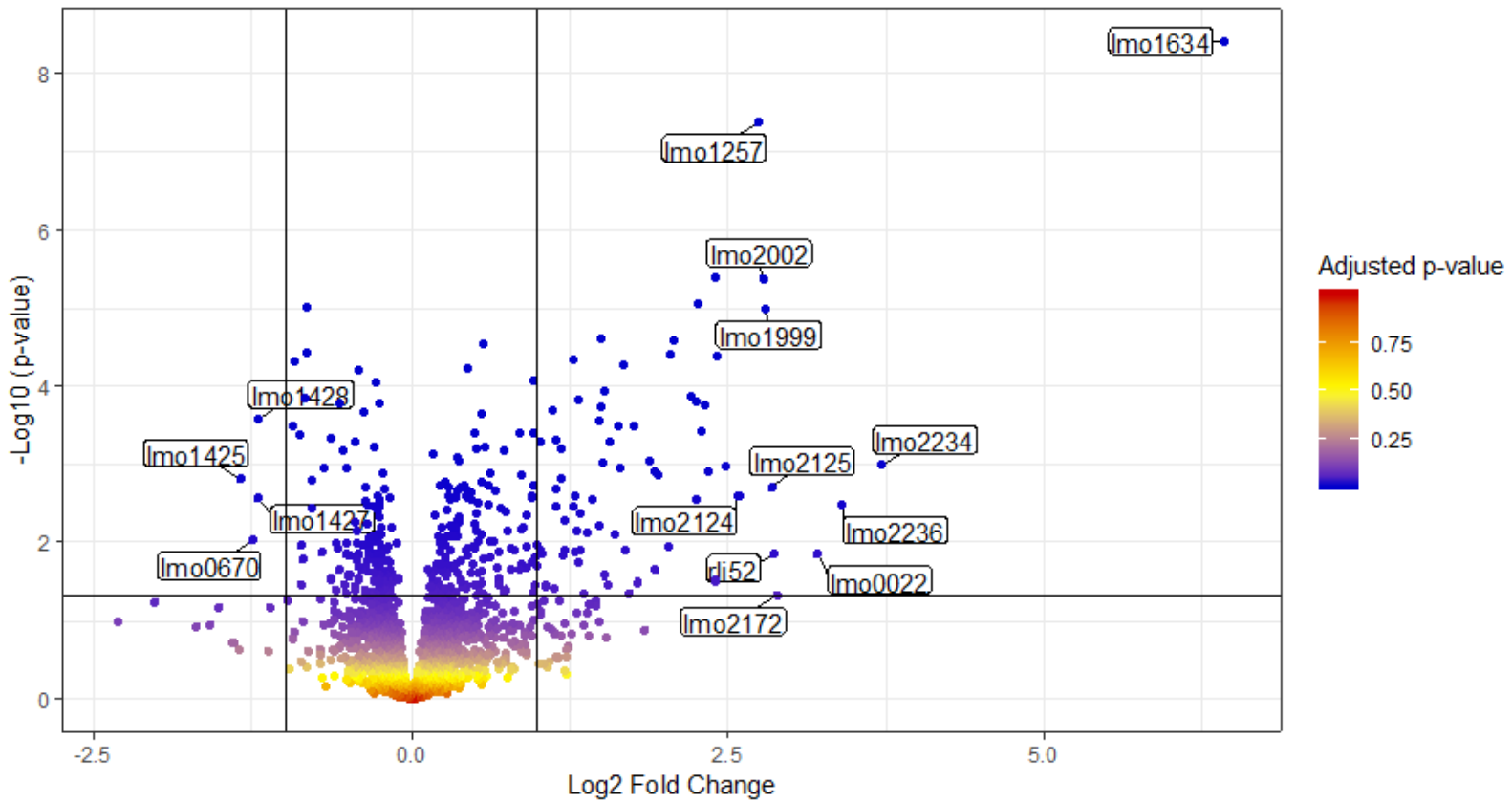

Figure 4. 5 The absence of Cys56 reduces the number of genes with significantly altered expression in the presence of visible light to 77.

RNA was sampled from exponentially growing cells exposed to either 0.6 mW cm^{-2} 470 nm light or darkness for 20 min at 30°C. Gene transcription was measured by RNA seq. and differential gene expression and p-values were determined using DESeq2.

To investigate whether σ^B-dependent changes in gene transcription were occurring in the C56A mutant strain in response to light, a heat map showing those genes that were most affected by light in the wild-type was generated and compared to the changes observed in the other conditions. Almost all genes showing a change in transcription $>\log_2 = 4$ in response to light in the wild-type were unaffected by light in the Δ*sigB* mutant. This is clearly seen on the heat map when the Δ*sigB* mutant and wild-type were compared to each other after light exposure (Fig. 4.6, *ΔsigB* Light vs. Wild-Type Light); this comparison reveals an inverse relationship in the direction of the effect compared to effect of light on the wild-type (Wild-Type Light vs. Wild-Type Dark). There were two exceptions to this trend; *rli18* and *rli62*, both of which encode sRNAs, were induced by light independently of σ^B (Fig. 4.6 *ΔsigB* Light vs. Wild-Type Light). The C56A mutant strain also showed this inverse relationship, with almost all of the light-affected genes showing the opposite effect in this strain to the wild-type (Fig. 4.6, *lmo0799* C56A Light vs. Wild-Type Light). Interestingly a comparison of the C56A mutant to the Δ*sigB* mutant in the presence of light revealed that the C56A mutant retained an altered pattern of gene expression in response to light, albeit not to the same extent as the wild-type (Fig. 4.6, *lmo0799* C56A Light vs. Δ*sigB* Light). This suggests that although the response to light is attenuated in a mutant lacking a functional Lmo0799 light sensor, σ^B-dependent changes in gene transcription still occur albeit to a reduced extent.

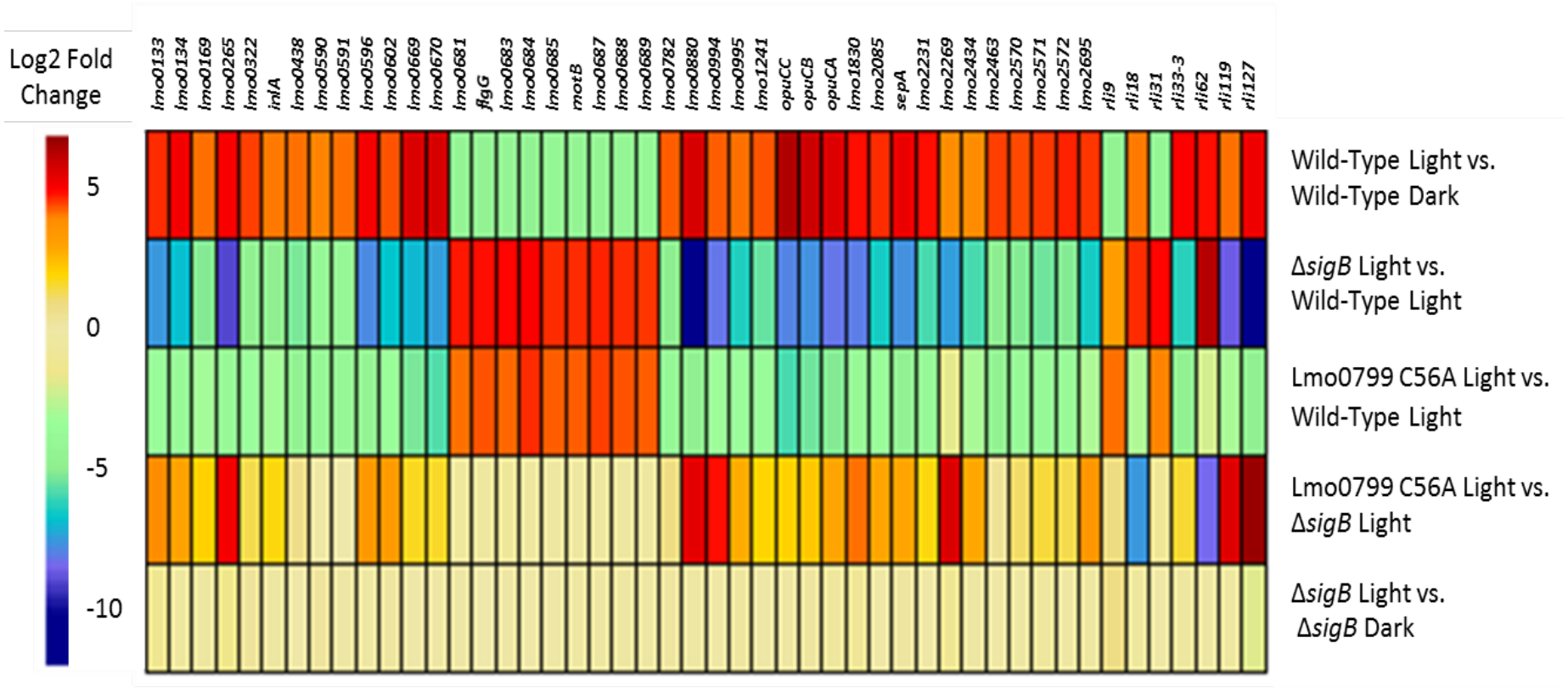

Figure 4. 6 SigB is required for the altered transcription of 98% of genes showing a greater than 16-fold change in transcription in response to visible light.

Genes showing a greater than 16-fold change in transcription in the wild-type in response to visible light were selected. All genes except for *rli18* and *rli62* require SigB to significantly alter their transcription in response to visible light.

4.3.6 RT-PCR confirms the requirement for Cys56 to alter the transcription of genes under the control of SigB

The final part of this study utilised RT-PCR to confirm the requirement for Lmo0799 Cys56 in significantly altering the transcription of *sigB* and the two SigB-dependent genes *lmo2230* and *opuCA.* The changes in transcription of all three genes was measured over a 30 min period, providing data 10 min before and after the 20 min time point chosen for RNA seq analysis. In agreement with the RNA seq analysis, the changes in transcription of *sigB* and the two σ^B-dependent genes, *lmo2230* and *opuCA,* in the presence of blue light were abolished in the absence of Lmo0799 Cys56 (Fig. 4.7). For all three genes, the isogenic parental strain showed statistically significant (p = ≤0.05) increased transcription compared to the C56A mutant strain. In agreement with the RNA seq analysis, RT-PCR showed the wild-type strain having 2, 4 and 5-log2 fold changes in *sigB, lmo2230* and *opuCA*, respectively, compared to the dark control. Transcription of these genes were also compared between the two strains in the absence of blue light, and no statistically significant (p = >0.05) differences were detected (Fig. 4.7). Thus, these results suggest that C56 is required for the activation of σ^B by blue light, though its absence does not affect σ^B in the absence of blue light.

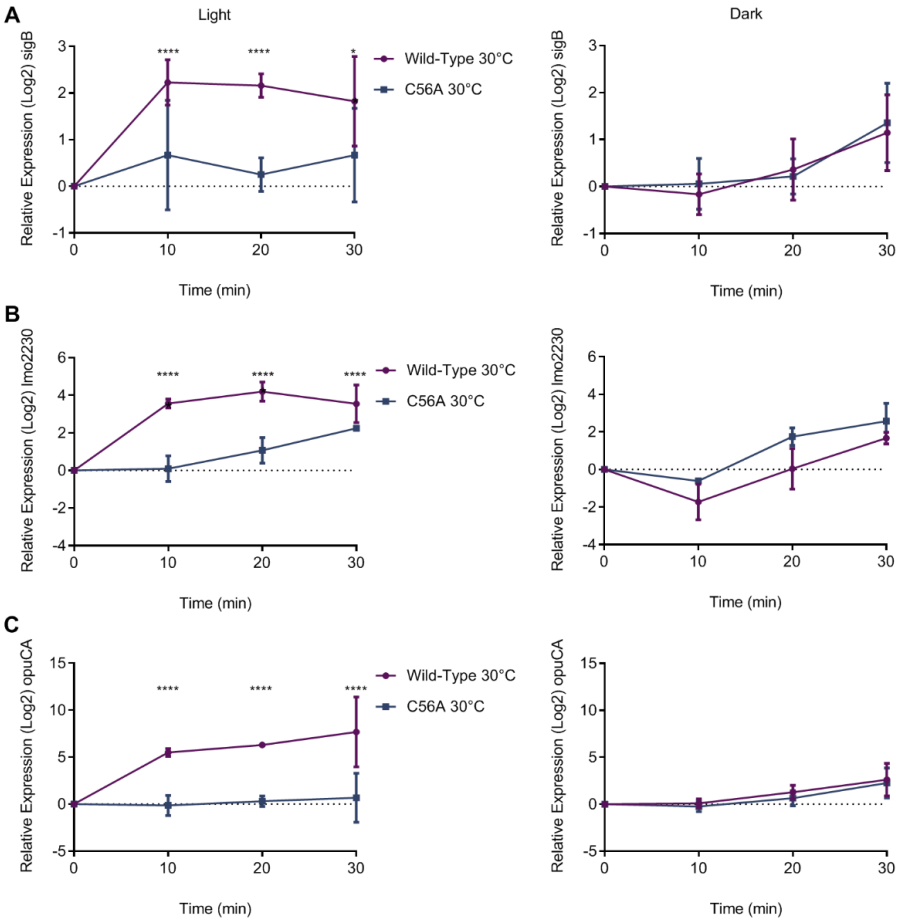

Figure 4. 7 Increases in the transcription of *sigB*, *lmo2230* and *opuCA* in the presence of visible light at 30°C is dependent upon Cys56 in Lmo0799.

Exponentially growing cells were exposed to 0.6 mW cm^{-2} 470 nm light for 30 min at 30°C or 37°C, with RNA samples collected at 10 min intervals. Relative transcription of *sigB* (A), *lmo2230* (B) and *opuCA* (C) was measured by RT-PCR. (****: p = ≤ 0.0001).

4.4. Discussion

4.4.1 Lmo0799 Cys56 is not required for resistance to killing by blue light

In the present study, SigB was found to play a pivotal role in controlling the transcriptional response to blue light, whereas the conserved cysteine residue of the blue light receptor Lmo799, the only know light sensor in *L.*

monocytogenes, was found to contribute to a much lesser extent. The requirement for SigB in blue light tolerance is in agreement with our previous study (O'Donoghue *et al.* 2016), however the increased dose of light administered in the current study (1008 J/cm^2 compared to 346 J/cm^2) suggest that this effect is independent of dose. While it is unusual that the loss of the conserved cysteine residue in Lmo0799 does not alter resistance to killing by blue light, it is possible that *L. monocytogenes* is able to sense and respond to secondary stresses associated with blue light exposure via an alternative mechanism. The primary bactericidal mechanism of blue light is thought to be through the production of reactive oxygen species (Lavi *et al.* 2004), with the addition of ROS scavengers to the medium capable of mitigating the inhibitory action of blue light on the growth *L. monocytogenes* (O'Donoghue *et al.* 2016). In the absence of the conserved cysteine residue in Lmo0799, *L. monocytogenes* may sense and respond to ROS via an alternative sensory mechanism.

4.4.2 Blue light exposure represses transcription of *L. monocytogenes* motility genes

Whole genome transcriptomics was performed on *L. monocytogenes* wild-type cells cultured in the presence of low intensity blue light or darkness for 20 min. Compared to the dark control, blue light exposure altered the transcription of over 600 genes (Fig. 4.2). A large proportion of these genes had previously been shown to be SigB-dependent, suggesting that transcription of the SigB regulon is expressed in response to blue light. This result was not unexpected, as previous studies have shown that exposure to blue light increases the transcription of several SigB-dependent genes (Ondrusch and Kreft 2011, Tiensuu *et al.* 2013). Interestingly, a previous study by Uesugi *et al.* (2016) did not detect significant upregulation of any genes after the exposure of *L. monocytogenes* to pulsed light of wavelength

greater than 400 nm. The difference between the results of the two studies may be due to the increased dose of light in the current study (4200 J/m^2) compared to the previous study (3.3 J/m^2), suggesting that the effect of blue light exposure on gene transcription is dose dependent. Moreover, the study by Uesugi *et al.* (2016) used pulsed light filtered to removed UV wavelengths, therefore exposing the bacteria to light across the entire visible spectrum, rather than just 470 nm as in the current study.

An analysis of the functional categories of genes affected by blue light identified a large proportion of downregulated genes that were involved in cell motility and chemotaxis. Previous studies have shown that blue light exposure inhibits cell motility (Ondrusch and Kreft 2011, Tiensuu *et al.* 2013, O'Donoghue *et al.* 2016), so a downregulation of genes involved in motility offers an explanation of the molecular mechanism for this response. A SigB promoter is located upstream from MogR, a transcriptional repressor of motility genes, (Toledo-Arana *et al.* 2009), so the downregulation of motility genes in the presence of blue light is likely to be due to increased SigB activity leading to increased expression of MogR, and therefore increased repression of motility gene transcription. The decrease in motility gene transcription mirrors the results of the previous study by Uesugi *et al.* (2016), suggesting that a lower dose is required to trigger this response than is required to increase gene transcription. While the physiological advantage for this phenotype is unknown, the inhibition of motility by blue light is not specific to *L. monocytogenes* and has been shown in both *E. coli* and *Salmonella typhimurium* (Taylor and Koshland 1975). Indeed, it would seem that a lack of motility in the presence of blue light could be deleterious to the bacterium as it would be unable to migrate to a dark environment, however decreased motility may be an energy-saving mechanism. The initiation of the general stress response is an energy intensive process for the cell, as is motility, so the inhibition of motility by the general stress

response may enable the cell to conserve energy for use in alternative protective and homeostatic processes.

In the absence of SigB, alterations in transcription were limited to ten genes, emphasising the dominance of SigB in the response to blue light. As expected, no genes involved in cell motility showed significant changes in transcription in response to blue light in the Δ*sigB* background, confirming that changes in motility gene transcription are SigB-dependent. In previous studies investigating the effects of blue light on cell motility, the Δ*sigB* mutant has shown no changes in motility in response to blue light (Ondrusch and Kreft 2011, Tiensuu *et al.* 2013, O'Donoghue *et al.* 2016). The current study provides molecular evidence that SigB is required for the inhibition of motility in response to blue light. The reduction in the number of significant changes in gene transcription from 600 to 10 genes in response to blue light exposure in the absence of SigB likely explains the increased sensitivity of the Δ*sigB* mutant to killing by blue light. It seems likely that genes belonging to the SigB regulon contribute to protection and repair functions that help to mitigate the damaging effects of blue light.

As with the Δ*sigB* mutant, the Lmo0799 C56A mutant showed a reduction in number of genes showing significant changes in transcription; just 77 genes were affected in this strain in response to blue light exposure compared to 600 in the wild-type. When compared to the wild-type, no genes showing significant up or down-regulation of transcription in the Lmo0799 C56A mutant mirrored the changes seen in the isogenic parental strain after exposure to blue light. However, when the Lmo0799 C56A mutant was compared to the Δ*sigB* mutant after exposure to blue light, 75% of genes that showed a significant change in gene transcription in response to blue light showed the same trend in transcriptional change as the wild-type in

response to blue light, albeit to a lesser extent (Fig. 4.6). Included in these genes showing an intermediate change in transcription in the Lmo0799 C56A mutant, were the highly SigB-dependent genes *opuCA* and *lmo2230*, suggesting that *L. monocytogenes* is able to activate SigB in response to blue light via an alternative mechanism that is independent of the light sensing capacity of Lmo0799. This intermediate effect may offer an explanation for the unexpected difference in resistance of the Lmo0799 C56A mutant compared to the Δ*sigB* mutant when challenged with a lethal dose of blue light exposure. This result also suggests that *L. monocytogenes* is able to sense and respond to secondary stresses associated with blue light exposure. This is not the first time that a level of redundancy has been associated with the different RsbR paralogues, the sensory proteins of the stressosome. A study on the responses of *B. subtilis* to ethanol stress utilising mutants lacking all bar one RsbR paralogue, found that all of the mutants were able to respond to ethanol stress, but the pattern and amplitude to which they responded to the stress varied (Cabeen *et al.* 2017). However, investigations into the activation of SigB by blue light via YtvA in *B. subtilis,* suggest that the deletion of *ytvA* abolishes SigB activation by blue light (Ávila-Pérez *et al.* 2006).

Interestingly, there were no significant differences in the transcription of motility genes, including the transcriptional repressor MogR, between the Lmo0799 C56A and Δ*sigB* mutants in response to blue light. This finding provides molecular evidence for the lack of motility repression in the Lmo0799 C56A mutant in response to blue light previously characterised (O'Donoghue *et al.* 2016), but also suggests that some SigB-dependent changes in gene transcription are dependent upon Lmo0799 Cys56, but others can be activated via an alternative mechanism. This result, and also the previous finding by Uesugi et al (2016) that motility can be repressed by a much lower dose of light than that required to increase the transcription

of other SigB-dependent genes, suggests that there may be a more refined level of regulation of the general stress response in *L. monocytogenes* than previously thought. Investigations into the activation of SigB in response to cold stress identified transcriptional changes in SigB-dependent genes in a Δ*rsbV* mutant, suggesting that SigB activation can occur independently of RsbV (Utratna *et al.* 2014). Therefore, it is possible that SigB can be activated in response to blue light via an alternative mechanism to Lmo0799 Cys56, but further work is needed to explain altered transcription of only some genes in the SigB regulon in the absence of Lmo0799 Cys56.

4.5 Conclusions

In conclusion, this study presents, to our knowledge, the first whole genome transcriptomic investigation into the response of *L. monocytogenes* to blue light in both the presence and absence of SigB and Lmo0799 Cys56. Through utilisation of the Lmo0799 C56A and Δ*sigB* mutants, the study has partially identified potential molecular roles for these proteins in light sensing and resistance. In addition, our results provide evidence that the inhibition of motility by blue light is due to increased SigB activity. Finally, the results of this study suggest that the SigB regulon can be partially activated by blue light in a way that does not depend on the light sensing functions of Lmo0799. The finding that some SigB regulon genes are unaffected in the Lmo0799 C56A mutant suggests that there may be a degree of selectivity to the general stress response in response to blue light.

Chapter 5: The Characterisation of RsbR and its Paralogues as Stress Sensors

5.1 Abstract

The stressosome complex is considered to be the stress sensing signalling hub of *Listeria monocytogenes,* yet little is known about the stresses that it senses. In the current study, work was carried out to identify the roles of the individual RsbR and paralogue proteins in stress sensing and virulence. Through the phenotypic, transcriptomic and proteomic assessments using single deletion mutants, the role of the individual RsbR proteins in growth, light and H_2O_2 stress, virulence in the G*alleria mellonella* infection model, and in influencing the transcription and expression *rsbR, rsbS* and *rsbT.* While a role was found for the alternative sigma factor SigB, in growth and biofilm in defined media and growth in the presence of hydrogen peroxide, no clear role for any single RsbR paralogue was detected within the conditions of this study. These results suggest that a level of redundancy between the proteins exists, in line with what has been previously found in *Bacillus subtilis*. Finally, results from RT-PCR and Western blotting suggest that the deletion of a single paralogue does not alter the transcription or translation of the other proteins of the stressosome.

5.2 Introduction

The ability of microorganisms to sense and respond to changes in their environment is a requirement for their continued survival and presence in ever changing environments. The ability of the Gram positive bacterium *Listeria monocytogenes* to withstand high levels of various environmental stresses has been well studied, with a particular focus on the role of the alternative sigma factor SigB in tolerance to these stresses (Ferreira *et al.* 2001, Utratna *et al.* 2011, Zhang *et al.* 2011, NicAogáin and O'Byrne 2016). It is hypothesised that *L. monocytogenes* senses environmental stresses via

a 1.8 mDa complex designated the 'stressosome', leading to the activation of the SigB signalling cascade (Murray *et al.* 2005).

The stressosome is composed of three proteins categorised as **R**egulators of **S**igma **B** and known as RsbT, RsbS and RsbR (Marles-Wright and Lewis 2010). Upon exposure to a stress, the stress signal is thought to be detected by either RsbR or one of its four paralogues (Ondrusch and Kreft 2011), leading to the phosphorylation of both RsbR and RsbS and enabling RsbT to dissociate from the stressosome complex and interact with RsbU to activate the SigB signalling cascade (Chen *et al.* 2003). In the absence of Prli42, *L. monocytogenes* shows an increased sensitivity to oxidative stress and this correlates with a reduced level of SigB activity compared to the wild-type under these conditions. Additionally the mutant lacking Prli42 has a decreased survival in macrophages (Impens *et al.* 2017).

In addition to RsbR, four paralogues of RsbR exist within the stressosome complex and are designated Lmo0161, Lmo0799, Lmo1642 and Lmo1842 in *L. monocytogenes* (Heavin and O'Byrne 2011, Ondrusch and Kreft 2011, Impens *et al.* 2017). At present, Lmo0799 is the only paralogue to have been confirmed as a stress sensor, and is responsible for detecting blue light, with RsbR and the remaining paralogues hypothesised to be environmental stress sensors based upon their homology to RsbR and its paralogues in *Bacillus subtilis* (Ondrusch and Kreft 2011). In the absence of Lmo0799, *L. monocytogenes* is unable to activate SigB in response to blue light exposure (Ondrusch and Kreft 2011, Tiensuu *et al.* 2013, O'Donoghue *et al.* 2016). However, the functions of the remaining paralogues have remained elusive. The presence of distinct C-terminal PAS domains in all of the paralogues suggests that could each contribute a different sensory capacity to the stressosome although there is little experimental evidence to support this idea at present.

Investigations into the roles of RsbR and its paralogues in activating SigB in response to ethanol stress in *B. subtilis,* identified potential roles for all of the paralogues, with the exception of the blue light sensor YtvA, but suggest that the response is primarily mediated by RsbRA (Cabeen *et al.* 2017). In deletion mutants expressing only one RsbR paralogue, cells were still able to respond to ethanol stress, but their stress-response profiles varied (Cabeen *et al.* 2017). Unlike *L. monocytogenes, B. subtilis* does not sense nutritional stress via the stressosome complex. However, the substitution of RsbR in a *B. subtilis* stressosome with *L. monocytogenes* RsbR enabled the activation of SigB in response to nutritional stress (Martinez *et al.* 2010). Interestingly, this study also found that the activation of SigB in response to nutritional stress was able to occur in the absence of the *B. subtilis* RsbR, RsbRA and RsbRB proteins, suggesting that these proteins block the activation of SigB in response to nutritional stress (Martinez *et al.* 2010). The results of these studies considered together, suggest that RsbR and its paralogues may be involved in not only sensing the presence of environmental stresses, but also in coordinating a response that is appropriate for both the type and intensity of the stress encountered.

The aim of the study described in this chapter was to build upon previous research characterising the roles of RsbR and its paralogues as stress sensors in *L. monocytogenes* (O'Donoghue 2017). In this study, we present findings that suggest that while the deletion of SigB influences the growth of *L. monocytogenes* in chemically defined media (DM), it is unlikely that a single RsbR paralogue is responsible for this phenotype. In addition, we present data suggesting that the absence of SigB is beneficial for cells during growth in the presence of low levels of hydrogen peroxide, but this phenotype is unlikely to be due to a single RsbR paralogue. Finally, we demonstrate through RT-PCR and Western blotting, that the deletion of one RsbR

paralogue does not alter the expression of *rsbR* or *rsbS,* but the deletion of *rsbR* abolishes expression of *rsbR* and the translation of RsbS.

5.3 Results

5.3.1 WGS identifies a previously characterised SNP in *rsbV* of the Δ*rsbR* mutant

Previously characterised phenotypes associated with the deletion of *lmo1642* and *lmo1842* from the genome were found to be due to a secondary mutation occurring in RsbV (O'Donoghue 2017). In order to reconstruct these mutants, electrocompetent EGDe cells were transformed with the pMAD plasmid carrying the deletion cassette previously designed by O'Donoghue (2017) (Fig. 5.1). Briefly, the deletion casettes were made up of 300 bp located immediately upstream and downstream of the gene selected for deletion fused together. After transformation, the plasmid was integrated into the genome at 42°C, at this temperature the plasmid cannot replicate unless it is integrated into the genome (Fig. 5.2), and then excised from the genome along with the wild-type gene via continuous passaging. The excision of the wild-type gene and plasmid was confirmed via PCR (Fig. 5.3). After reconstruction of the Δ*lmo1642* and Δ*lmo1842* mutants, DNA extracts from each of the strains were sent for WGS, and results were analysed for SNPs by breseq (Deatherage and Barrick 2014). Breseq analysis confirmed, in addition to PCR, deletion of the target gene in each strain (Fig. 5.4), and identified any other SNP's compared to the isogenic parental strain. The previously detected I23T SNP mutation identified in the Δ*lmo1642* and Δ*lmo1842* mutants was predicted to interfere with the binding of RsbV to RsbU and RsbW at 37°C, leading to a reduction in SigB activity (O'Donoghue 2017). Analysis of the Δ*rsbR* mutant WGS in the current study identified this

same SNP mutation in the Δ*rsbR* mutant (Fig. 5.4). All other non-silent SNPs identified in the strains used in this study are listed in Table 5.1.

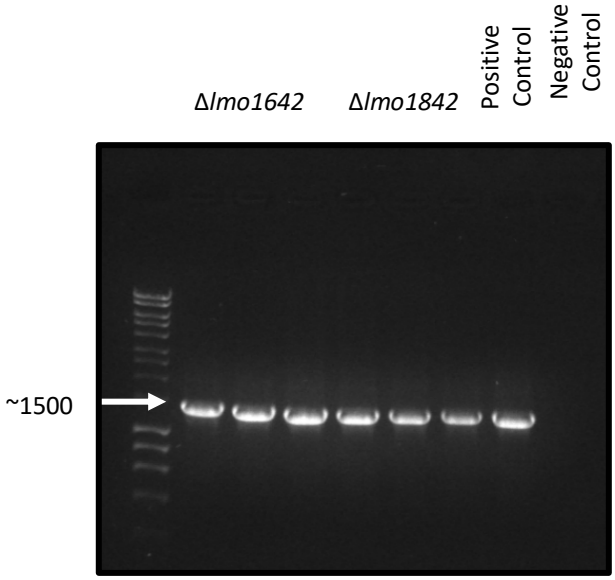

Figure 5. 1 PCR confirms the transformation of the wild-type cells with the pMAD plasmid.

Three colonies were randomly chosen from the electrocompetent cells that had undergone transformation and had been cultured on a BHI erythromycin plate. PCR primers targeting the *bgaB* region of the pMAD plasmid were used to confirm the presence of the plasmid in these cells. The purified plasmid was used as a PCR template for the positive control and sterile deionised water was used for the negative control.

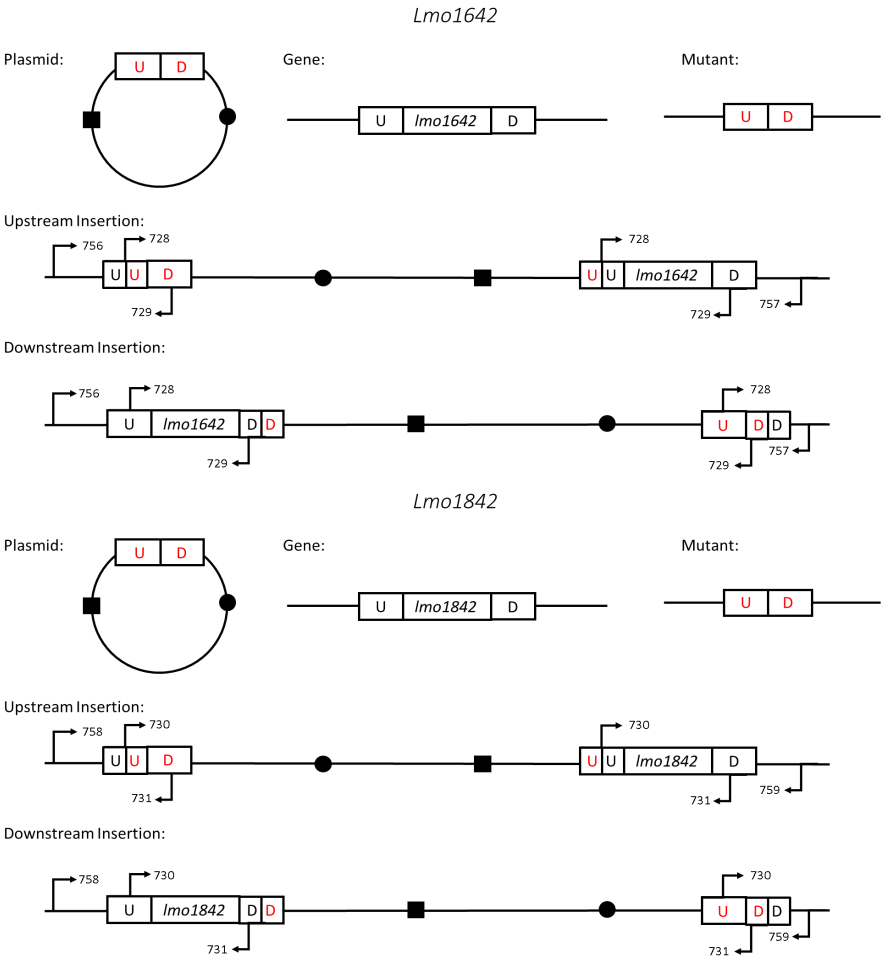

Figure 5. 2 Chromosomal integration of pMAD plasmid containing either the lmo1642 or lmo1842 deletion constructs.

Several transformants were selected and cultured in BHI broth at 42°C to initiate integration of the plasmid into the genome. The plasmid cassette is identical to the upstream (U) and downstream (D) flanking regions of the wild-type gene can integrate at either the upstream (U) or downstream (D) flanking region of the wild-type gene. PCR primers targeting regions internally of the gene or in the external flanking regions can be used in varying combinations to confirm upstream or downstream integration.

■ Erythromycin resistance cassette. ● Temperature-sensitive origin of replication.

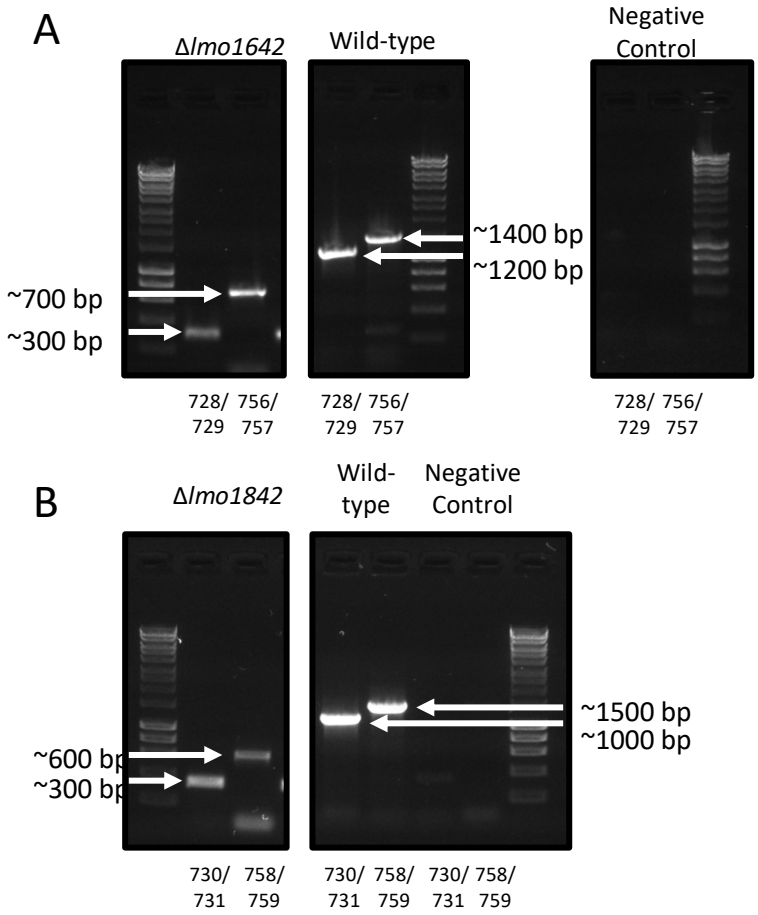

Figure 5. 3 PCR confirms the deletion of *lmo1642* or *lmo1842* from the genome.

Five colonies were randomly chosen from the integrated cells and passaged every 12 h at 30°C for 5 days in BHI broth. At each passage, 100 µl of culture was spread onto BHI agar with and without 2 µg/mL erythromycin and incubated at 30°C. Colonies were grid-plated from the BHI agar plate without erythromycin onto BHI plates with and without erythromycin, and colonies showing erythromycin sensitivity were checked for the presence of the mutation by PCR using primers targeted to the internal and external regions of the (A) *lmo1642* and (B) *lmo1842* genes. A wild-type colony was used as a positive control and water for the negative control.

Table 5. 1. Reporting of SNPs for all strains used in this study.

Strain	Mutation	Annotation	Gene	Description
Δ*rsbR*	Δ837 bp		rsbR	RsbR
	T → C	I23T (ATC → ACC)	rsbV	Anti-Sigma-B factor antagonist
	G → A	E280K (GAG → AAG)	gatD_2 →	Galactitol-1-phosphate 5-dehydrogenase
	A → G	C354R (TGC → CGC)	recQ_2 ←	ATP-dependent DNA helicase RecQ
	A → T	K547N (AAA → AAT)	Lmo0159 →	Peptidoglycan-binding protein
	G → A	G93S (GGC → AGC)	atpF →	ATP synthase subunit b
	C → T	W7* (TGG → TGA)	mall_2 ←	Oligo-1,6-glucosidase
Δ*lmo0161*	G → A	W324* (TGG → TGA)		
	Δ831 bp		*Lmo0161* ←	RsbT co-antagonist protein Lmo0161
Δ*lmo0799*	Δ766 bp		lmo0799	Lmo0799
	A → G	C354R (TGC → CGC)	recQ_2 ←	ATP-dependent DNA helicase RecQ
Δ*lmo1642*	A → C	Y89D (TAT → GAT)	*Lmo1535* ←	Putative transcriptional regulatory protein
	Δ804 bp		*Lmo1642* ←	RsbT co-antagonist protein Lmo1642
Δ*lmo1842*	Δ825 bp		Lmo1841 → / ← *lmo1843*	Hypothetical protein / Ribosomal large subunit pseudouridine synthase D
	(A) $_{7 \rightarrow 8}$		comK →	Competence transcription factor
	Δ77 bp		dnaX_1 ← / ← lmo2705	DNA polymerase III subunit tau / DUF3284 domain-containing protein

ǂSNPs were detected by analysing the WGS results using breseq (Deatherage and Barrick 2014).

5.3.2 The deletion of RsbR or its paralogues decreases the exponential growth rate under some conditions

The Δ*sigB* mutant has been shown in previous experiments to have reduced exponential growth rate in DM supplemented with glycerol (Abram, Starr, *et al.* 2008). To investigate the potential roles of RsbR and its paralogues in this SigB-dependent phenotype, growth experiments in DM supplemented with either glucose or glycerol were conducted. In the presence of glucose, all of the deletion mutants, with the exception of Δ*lmo1642* at 30°C and Δ*lmo0799* at 37°C, showed a significantly (p = ≤0.05) reduced growth rate compared to the isogenic parent strain (Fig. 5.4). When the exponential growth rate was measured in DM supplemented with glycerol at 30°C, all deletion mutants except for Δ*lmo1642* had a small but significant (p = ≤0.05) reduction in growth rate compared to the wild-type (Fig. 5.4). In the presence of glycerol at 37°C, only the Δ*sigB* and Δ*rsbR* mutants had a significantly (p = ≤0.001) reduced growth rate compared to the wild-type (Fig 5.4). When considered together, the data reconfirm the role for SigB in the utilisation of glycerol, but suggest that none of the RsbR paralogues play a crucial role in growth when either glucose or glycerol is the sole carbon source.

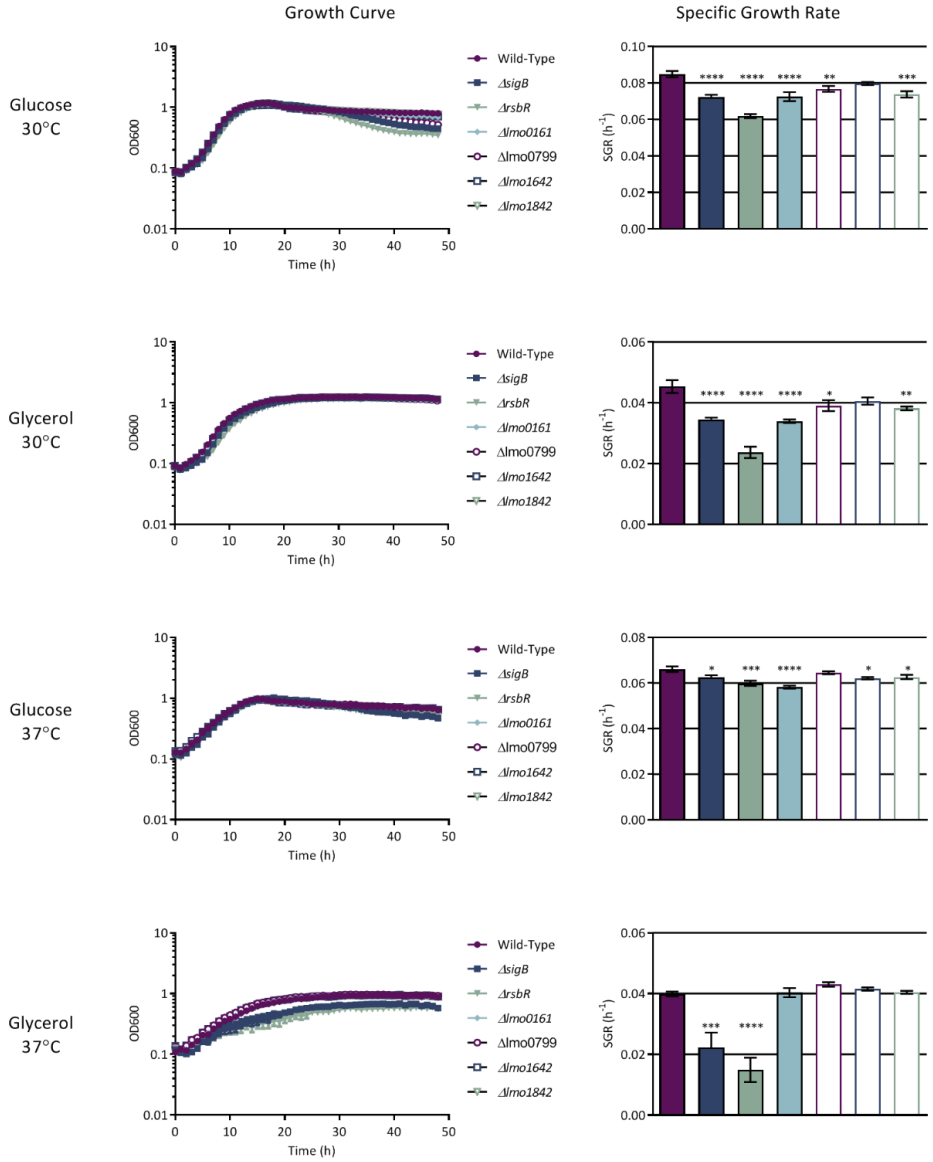

Figure 5. 4 The deletion of RsbR or one of its paralogue alters the exponential growth rate in DM supplemented with either glucose or glycerol at 30°C or 37°C.

Cultures were grown to stationary phase in BHI Broth at the appropriate temperature and diluted to OD ~0.1 in DM supplemented with either glucose or glycerol (0.4% (v/v)). Cultures were grown for 48 h in a Bioscreen C at the appropriate temperature with continuous shaking and OD was recorded at 1 h intervals. Specific growth rate was calculated using the exponential growth equation between 5 h and 8 h of growth. Data are the mean and SD of two independent replicates, each with three technical replicates. Statistical significance was determined between the wild-type and the mutants using a ANOVA with Dunnett's test to correct for multiple comparisons. (*: $p = \leq 0.05$; **: $p = \leq 0.01$ ***: $p = \leq 0.001$; ****: $p = \leq 0.0001$).

5.3.3 The deletion of SigB or RsbR reduces biofilm formation in DM supplemented with glucose at 30°C

A role for SigB in both static and continuous flow biofilm formation has previously been shown (van der Veen and Abee 2010), possibly due to its role in regulating motility (Lemon *et al.* 2010). In the current study, a role for SigB and RsbR and its paralogues was investigated in biofilm formation in DM supplemented with either glucose or glycerol. In the presence of glucose at 30°C, the Δ*sigB* and Δ*rsbR* mutants produced significantly (p = ≤0.01) less biofilm than the wild-type (Fig. 5.5). Under these conditions, no other strains showed a significant (p = >0.05) change in biofilm formation compared to the wild-type (Fig. 5.5). When the strains were grown in the presence of glycerol at 30°C, or in the presence of either glucose or glycerol at 37°C, no deletion mutant showed significant (p = >0.05) changes in biofilm formation compared to the wild-type (Fig. 5.5). Overall, these data suggest that SigB and RsbR may be involved in biofilm formation at 30°C during growth in glucose.

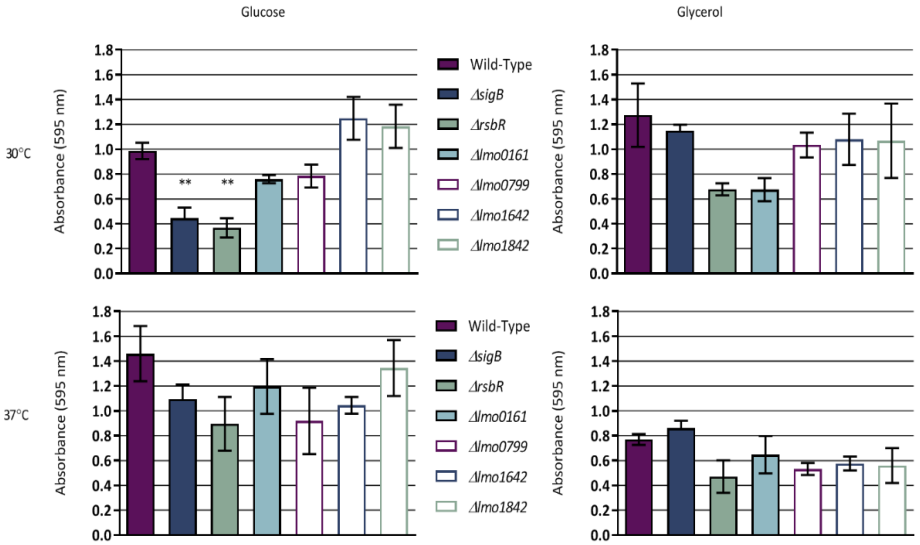

Figure 5. 5 The deletion of SigB or RsbR reduces biofilm formation in DM supplemented with glucose at 30°C.

Cultures were grown to stationary phase in BHI Broth at the appropriate temperature and diluted to OD ~0.1 in DM supplemented with either glucose or glycerol (0.4% (v/v)). Cultures were grown statically for 48 h in 96-well polystyrene plates, then biofilms were stained with crystal violet and destained with ethanol. Absorbance was read at 595 nm. Data are the mean and SD of two independent replicates, each with three technical replicates. Statistical significance was determined between the wild-type and the mutants using a ANOVA with Dunnett's test to correct for multiple comparisons. (**: p = ≤0.01).

5.3.4 The deletion of RsbR or its paralogues does not alter the sensitivity of *L. monocytogenes* to blue light

Previously, we have shown a role for SigB, but not the RsbR paralogues, in blue light tolerance at 30°C following growth in BHI medium (O'Donoghue *et al.* 2016). The present study investigated the roles of SigB and the RsbR paralogues in blue light tolerance after growth in DM at both 30°C and 37°C, to test the effect of growth medium and temperature on the role of these proteins in blue light tolerance. Cells were exposed to 35 mW cm^2 470 nm light for 8 h, with survival measured at 2 h intervals. No significant (p = >0.05) differences in the number of survivors between the wild-type and the deletion mutants was detected at either of the temperatures (Fig. 5.6). The results of this experiment demonstrate that σ^B does not contribute to light

survival when cells are grown in DM, in contrast to the light sensitivity that has been reported for BHI-grown *L. monocytogenes* Δ*sigB* mutant cells (O'Donoghue et al).

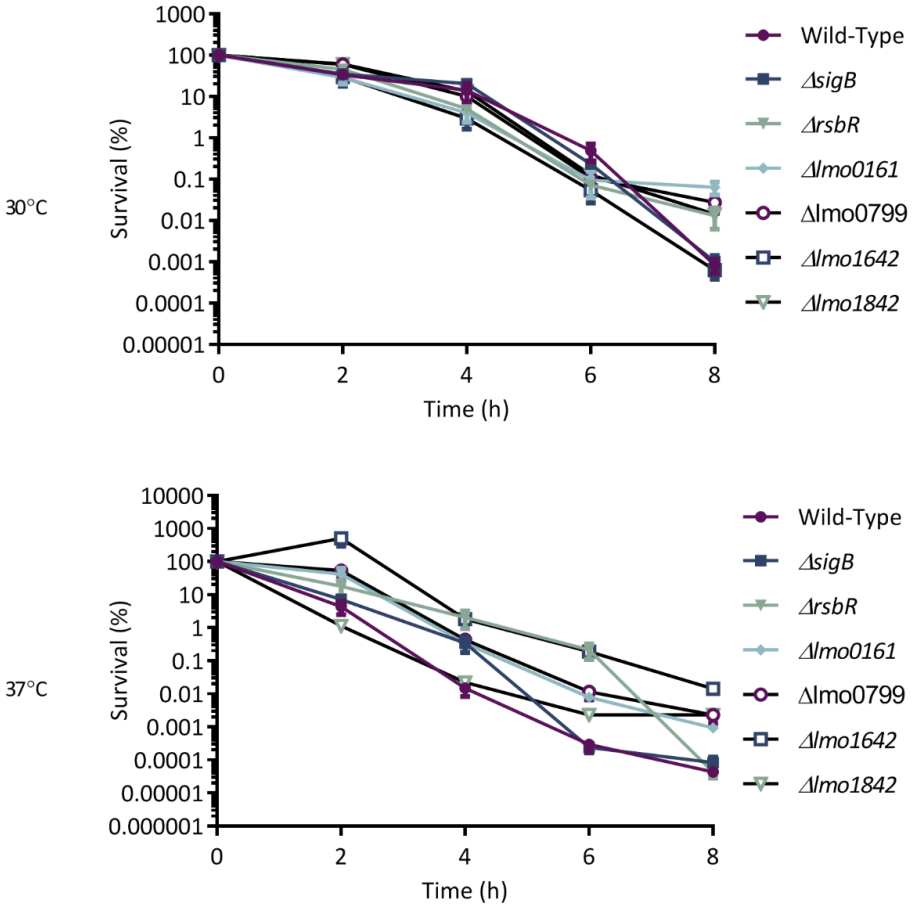

Figure 5. 6 The deletion of RsbR or its paralogues does not affect the tolerance of L. monocytogenes to visible light.

Cultures were grown to stationary phase at 30°C or 37°C in DM. Cells were centrifuged, washed once in PBS and resuspended in PBS to OD_{600} 1, then exposed to 35 mW cm^{-2} 470 nm light for 8 h. Cfu/mL were measured at 2 h intervals. Data represent the mean of two independent replicates, each with three technical replicates. No statistical significance was determined between the wild-type and each of the mutants using a one-way ANOVA with Dunnett's multiple comparisons test.

5.3.5 The deletion of SigB or RsbR is beneficial for growth in the presence of low levels of hydrogen peroxide

Previous work investigating the mechanism of bacterial inactivation by blue light, identified reactive oxygen species as a possible mediator (O'Donoghue *et al.* 2016). As we did not identify a role for SigB or RsbR and its paralogues in resistance to killing by blue light, the role of these proteins in growth in the presence of low level reactive oxygen species initiated by 0.045% (v/v) H_2O_2 was examined. Overnight cultures were diluted to OD ~0.1 and incubated for 32 h, with growth measured at 2 h intervals. At 30°C, the Δ*sigB* mutant had reached a significantly (p = ≤0.001) higher OD than the wild-type after 26 h, and this difference remained for the duration of the experiment (Fig. 5.7). At 37°C, the Δ*sigB* mutant reached a significantly (p = ≤0.0001) higher OD than the wild-type after 20 h of growth, and the Δ*rsbR* mutant also had significantly (p = ≤0.01) more growth after 22 h (Fig. 5.7). Though not statistically significant, the wild-type and other mutants showed growth after 24 h at 37°C but not at 30°C, suggesting that there is an effect of temperature on the ability of these strains to respond to reactive oxygen species. Overall, these results suggest that the presence of SigB during growth in low levels of hydrogen peroxide is detrimental to cells at both 30°C and 37°C.

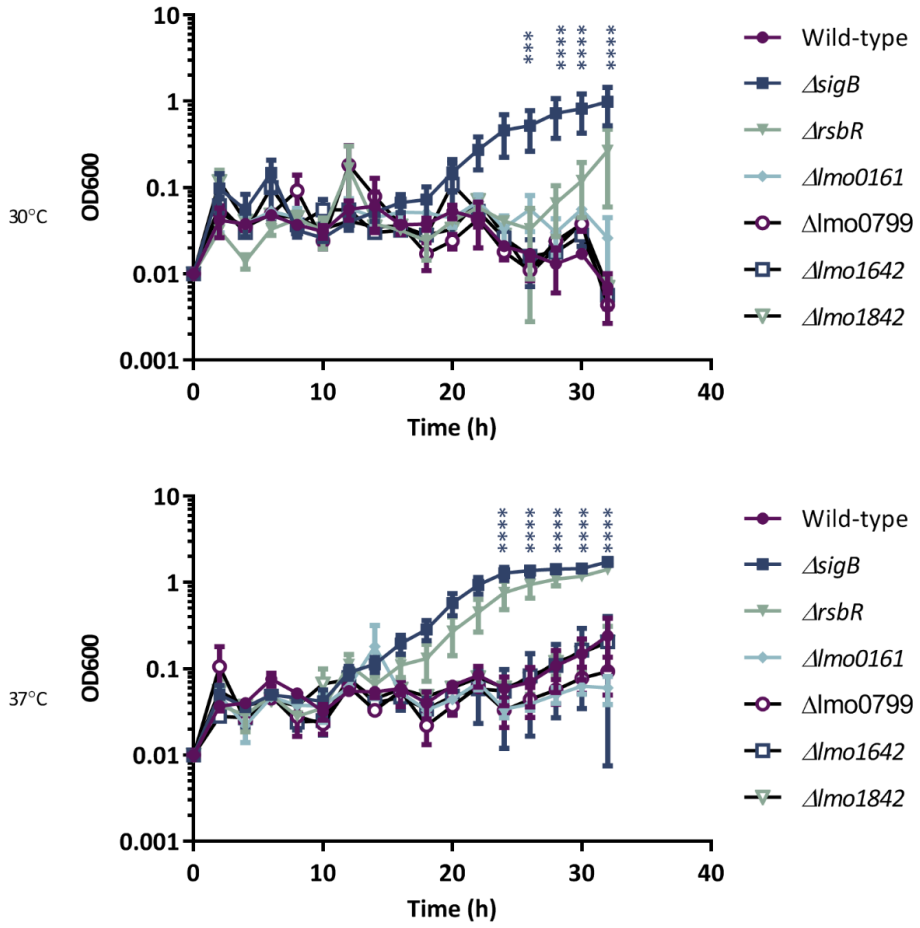

Figure 5. 7 The absence of SigB is beneficial to cells during growth in 0.045% H2O2.

Cultures were grown to stationary phase for ~20 h at 30°C or 37°C in DM and diluted to OD 0.2 in fresh medium supplemented with 0.045% H_2O_2. Cultures were grown at the appropriate temperature in darkness for 32 h and the OD was measured at 2 h intervals. The data show the mean and SD of three biological replicates. Statistical significance was determined between the wild-type and the mutants using a two-way ANOVA with Dunnett's multiple comparisons test. (***: p = ≤ 0.001; ****: p = ≤ 0.0001 in the Δ*sigB* and Δ*rsbR* mutants).

5.3.6 The deletion of RsbR or its paralogues does not affect the virulence of *L. monocytogenes* in the *Galleria mellonella* virulence model

In addition to its role in the general stress response, SigB has been implicated in the virulence of *L. monocytogenes* (Kazmierczak *et al.* 2003), both

dependent and independently of PrfA (Nadon *et al.* 2002, Garner *et al.* 2006). The virulence of the wild-type and deletion mutant strains was assessed in the *G. mellonella* virulence model (Joyce and Gahan 2010) in order to investigate their role in virulence. *G. mellonella* larvae were inoculated with stationary phase cultures grown at 30°C and 37°C, with the Δ*prfA* mutant used as an avirulent control (Chakraborty *et al.* 1992). At 30°C, the wild-type demonstrated significantly (p = ≤0.001) more killing than the Δ*prfA* mutant control 3 days after inoculation (Fig. 5.8). In addition, the wild-type caused significantly (p = ≤0.05) more killing than the Δ*sigB,* Δ*lmo0799* and Δ*rsbR* mutants 3 days after inoculation (Fig. 5.8). From day 4 post-inoculation, the wild-type only had significantly (p = ≤0.05) more killing than the Δ*sigB* mutant, which lasted until day 5 post-inoculation (Fig. 5.8). At 37°C, the wild-type demonstrated significantly (p = ≤0.05) more killing than the Δ*prfA* mutant 4 days' post-inoculation, but did not show any significant (p = >0.05) differences in virulence compared to any of the other deletion mutants (Fig. 5.8). Overall, these results suggest that SigB may play a short-term role in virulence after incubation at 30°C, but not at 37°C. Furthermore the data indicate that none of the RsbR paralogues are solely responsible for this contribution of σ^B to virulence.

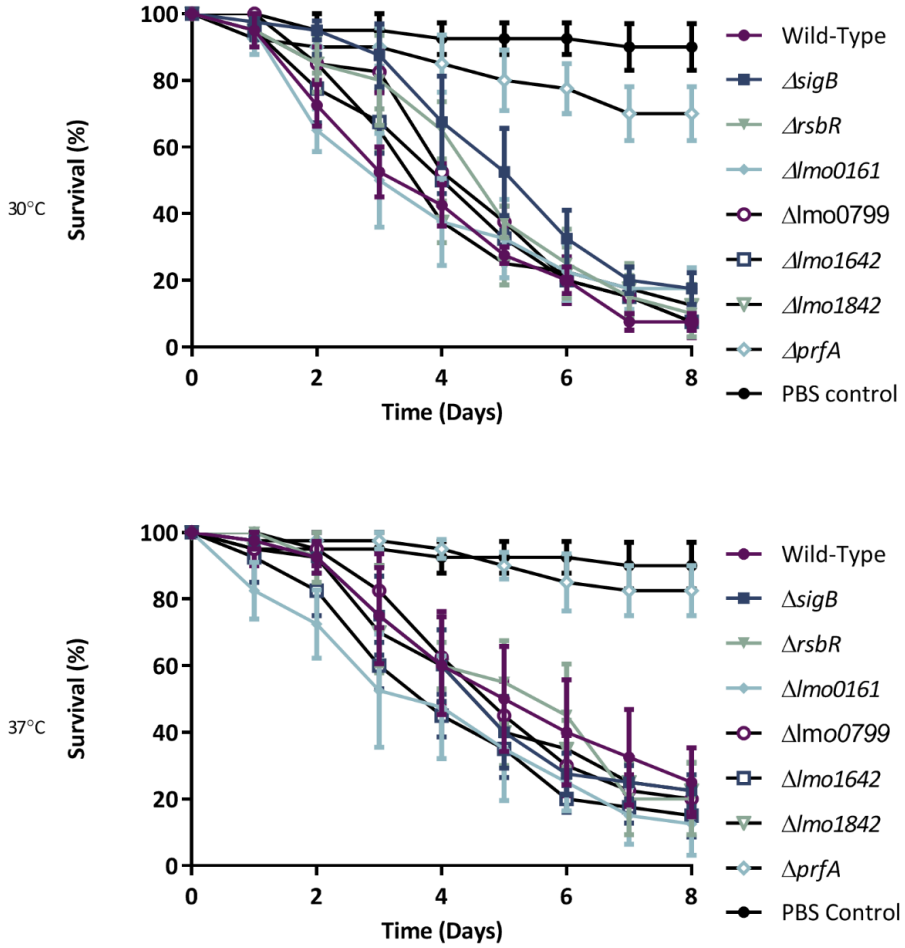

Figure 5. 8 The deletion of RsbR or its paralogues does not affect the virulence of *L. monocytogenes* in the *Galleria mellonella* virulence model.

Cultures were grown to stationary phase for ~16 h at 30°C or 37°C in BHI broth, washed once in PBS and resuspended to OD_{600} 0.1 in PBS. Ten microliters were inoculated into the second last pseudopod of the *G. mellonella* larvae and larvae were incubated at 37°C. Mortality was recorded at 24 h intervals for 8 days. Ten larvae were used in each biological replicate, and four biological replicates were conducted. No statistical significance was determined between the wild-type and the mutants using a two-way ANOVA with Dunnett's test to correct for multiple comparisons.

5.3.7 The deletion of RsbR reduces the levels of both RsbR and RsbS compared to the wild-type

The final part of this study investigated the effect of the targeted gene deletions on the translation of RsbR and RsbS, and the transcription of *rsbR, rsbS* and *rsbT.* Reanalysis of the Δ*rsbR* mutant identified the deletion of the Shine-Dalgarno region of *rsbS* in addition to *rsbR.* As a result of this, it is unlikely that *rsbS* can be translated. Secondly, the effect of the SNP in *rsbV* in the Δ*rsbR* mutant on transcription and translation on the stressosome components is unknown. The analysis of RsbR and RsbS levels in protein extracts from stationary phase cells by Western blot analysis using anti-RsbR and anti-RsbS antibodies, indicated that the deletion of *rsbR* abolished RsbR expression as expected, and reduced RsbS levels compared to the wild-type at both 30°C and 37°C (Fig. 5.9). The deletion of any of the other genes did not alter the levels of RsbR or RsbS compared to the wild-type at either temperature (Fig. 5.9). The transcription of *rsbS, rsbT* and *rsbR* was measured in exponential phase cells by RT-PCR. As expected, the Δ*rsbR* mutant showed no transcription of *rsbR* at either temperature (Fig. 5.10C), and therefore the relative expression of *rsbR* in the Δ*rsbR* mutant compared to the wild-type could not be calculated. The deletion of any of the target genes did not significantly (p = >0.05) alter the transcription of *rsbS, rsbT* or *rsbR* at 30°C or 37°C (Fig. 5.10). The results of these experiments suggest that the deletion of *rsbR* abolishes both the transcription and translation of *rsbR*, and reduces the translation of RsbS.

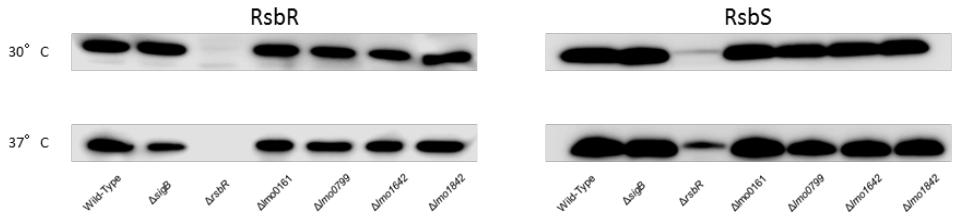

Figure 5. 9 The deletion of *rsbR* reduces the levels of both RsbR and RsbS compared to the wild-type as determined by Western blot analysis.

Cells were grown for ~16 h in Brain Heart Infusion broth at 30°C or 37°C with shaking in darkness. Protein was extracted from the cells and standardised to 0.55 mg/mL in sample buffer. Thirteen micrograms of protein were loaded on to 16% glycine gel (RsbR) and 12% tricine gel (RsbS) and separated by SDS-PAGE. Protein was transferred onto PVDF membrane and incubated with antibodies targeted against RsbR and RsbS. Western blots were imaged via chemiluminescence on a LICOR Odyssey Fc machine.

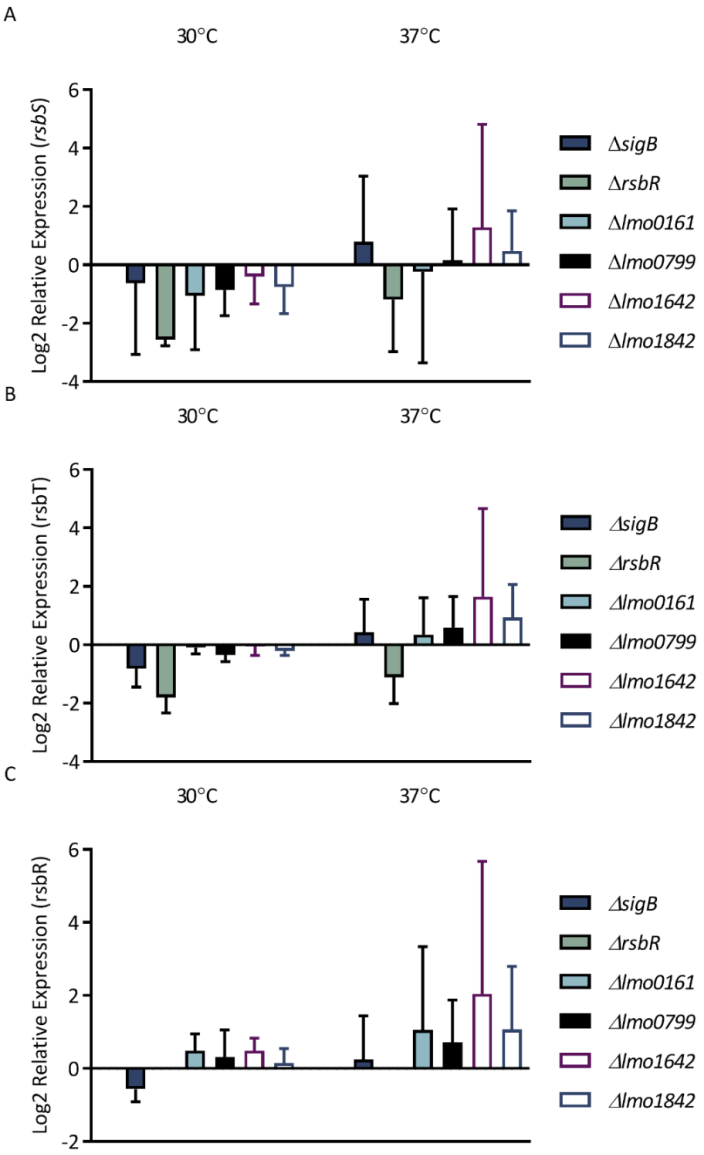

Figure 5. 10 The deletion of *rsbR* or its paralogues from the genome does not alter the transcription of *rsbR*, *rsbS* or *rsbT* at 30°C or 37°C.

Cultures were grown to OD_{600} ~0.2 in BHI broth at 30°C and 37°C, in darkness. RNA was extracted from samples and converted to cDNA. RT-PCR was carried out against *rsbS*, *rsbT,* and *rsbR*. The results from the RT-PCR analysis were normalised using *16S* as an internal standard, and relative expression levels between the wild-type and the mutants were calculated using the method described by Pfaffl (2001) on Microsoft Excel. No statistical significance was

5.4 Discussion

5.4.1 The construction of deletion mutants gives rise to secondary mutations

In this study, Δ*lmo1642* and Δ*lmo1842* mutants were constructed to create a mutant library with single knockout mutants for each of the RsbR paralogues. This mutant library was used to assess the role of each of these paralogues in the response of *L. monocytogenes* to a selection of stresses where a role for SigB has previously been shown. WGS was employed to confirm the presence of the desired genetic mutation in each strain, and also to detect any secondary mutations that may have arisen during the mutant construction process. WGS analysis of the Δ*rsbR* mutant identified the presence of a SNP in *rsbV* resulting in the conversion of an isoleucine to a threonine at position 23 (Table 5.1). This same SNP has previously been identified in deletion mutants constructed within our laboratory, and further investigations revealed that it was responsible for a reduction in SigB activity at 37°C, but had no effect on SigB activity levels at 30°C (O'Donoghue 2017). Strains containing the I23T mutation also showed increased sensitivity to acid stress compared to the wild-type at 37°C, attributed to the reduced SigB activity at this temperature (O'Donoghue 2017).

Our laboratory is not the first to report secondary mutations arising in the SigB operon during deletion mutant construction. Attempts by Quereda *et al.* (2013) to construct *L. monocytogenes* mutants lacking cell wall encoding genes resulted in secondary mutations occurring in *rsbS, rsbU* and *rsbV* that all resulted in reduced SigB activity and increased acid sensitivity compared to the wild-type. Similarly, the characterisation of 166 *L. monocytogenes* isolates in relation to cold, salt and acid stress resistance identified a number

of stress sensitive isolates with a premature stop codon in either *rsbS, rsbV* or *rsbU* (Hingston *et al.* 2017). Taken together, these studies indicate that secondary mutations in the SigB operon can arise during both the mutation process or spontaneously in the environment. While the exact mechanism for their common occurrence during the mutation process is unknown, it is known that the Δ*sigB* mutant has a growth advantage under certain conditions compared to the wild-type (Brøndsted *et al.* 2003, Chaturongakul and Boor 2004, O'Donoghue *et al.* 2016). Therefore, we hypothesise that the SNP within the SigB operon provides a growth advantage compared to the wild-type at a stage of the mutation generation process, in the case of our laboratory it almost certainly arose during the generation of the electrocompetent cells, increasing the likelihood of cells identified as having the desired mutation also carrying a secondary SNP within the SigB operon.

The results of this study identified a potential role for RsbR in growth in DM supplemented with glycerol at 37°C. Previously, a role had been identified for SigB in the utilisation of glycerol in DM at 37°C (Abram, Su, *et al.* 2008), and the results of our study confirmed this as the Δ*sigB* mutant displayed a significantly reduced growth rate compared to the wild-type. While it is tempting to speculate that the reduced growth rate of the Δ*rsbR* mutant is due to RsbR being involved in glycerol metabolism, it is most likely to be due to the reduced SigB activity at 37°C caused by the I23T SNP (O'Donoghue 2017). To confirm whether this is the case, the Δ*rsbR* mutant should either be reconstructed or complemented to determine which of the two mutations is responsible for this phenotype.

5.4.2 The deletion of a single RsbR paralogue does not alter the resistance of *L. monocytogenes* to environmental stress

The second part of this study focused upon the potential roles for RsbR and its paralogues in blue light stress and oxidative stress, and virulence. A role for SigB in blue light tolerance has been demonstrated when cells have been cultured in BHI (O'Donoghue *et al.* 2016), however BHI is a complex medium that can be variable between batches. In the current study, DM was used to determine whether the role of SigB in blue light resistance was a phenotype that was not specific to growth in BHI. The results of this study suggest that SigB does not play a significant role in light resistance after growth in DM. However, when the roles of SigB, RsbR and the RsbR paralogues in growth in the presence of mild oxidative stress were examined, the results suggest that the absence of SigB is beneficial to the cells. This result was not entirely unexpected as previous work by Boura *et al.* (2016) demonstrated that the loss of SigB induced a resistant phenotype in *L. monocytogenes* to the presence of hydrogen peroxide after growth in BHI. In a previous study investigating the growth of *L. monocytogenes* in the presence of low levels of hydrogen peroxide in BHI broth, the wild-type was able to grow in the presence of 0.045% hydrogen peroxide (Seifart Gomes *et al.* 2011). In the current study, the wild-type was unable to show a significant increase in growth over the time course of the experiment, though an increase in growth was detected after 24 h at 37°C, suggesting that the presence of hydrogen peroxide in DM is more stressful for the cells than it is in BHI broth.

A role for SigB has been shown during the early stages of infection, with the Δ*sigB* mutant showing reduced invasion of human epithelial cells (Kim *et al.* 2005). In the current study, the *G. mellonella* infection model was used to assess the role of the RsbR paralogues in virulence. The results of our study suggest that SigB is not required for virulence in the *G. mellonella* infection

model, as the Δ*sigB* mutant did not show a significant reduction in virulence during the study. The results of our study confirm those of previous studies by Joyce and Gahan (2010), whose study also showed that this effect was independent of growth temperature, and Rakic Martinez *et al.* (2017). A limitation of both the current study and previous studies is the subcutaneous inoculation of the larvae as opposed to oral infection, as this allows the bacteria to bypass the gastrointestinal tract. Previous investigations into *L. monocytogenes* virulence has identified a requirement for the three SigB-dependent genes *inlA, inlB* and *opuC* during the gastrointestinal phase of infection (Sleator *et al.* 2001, Kazmierczak *et al.* 2003). Upon entry into gastrointestinal epithelial cells, expression of *inlA* and *inlB* becomes PrfA-dependent and SigB-independent (Toledo-Arana *et al.* 2009), suggesting that SigB is not required after bacterial exit from the gastrointestinal tract. Therefore, future studies investigating *L. monocytogenes* virulence in the *G. mellonella* model should consider an oral method of infection as opposed to the subcutaneous injection utilised in the current study.

The final part of this study aimed to investigate the effect of the *rsbR* paralogue gene deletions on the transcription and translation of *rsbR, rsbS* and *rsbT.* When the levels of RsbR and RsbS were measured in each of the mutants by Western blotting, no mutants showed altered levels of either protein compared to the wild-type, with the exception of the Δ*rsbR* mutant. The inability of the deletion of one stressosome protein to influence the levels of another suggests that there is no inter-regulation of the individual protein levels. It is also likely that the deletion of a single RsbR paralogue protein does not negatively influence the stability of the stressosome. Previous research into the structure of the stressosome suggested that while it is critical to have a 1:1 ratio of RsbS:RsbT to prevent continual SigB activation, the levels of RsbR are less important (Marles-Wright and Lewis, 2010). The reduced levels of RsbS in the Δ*rsbR* mutant are likely to be due

to the deletion of the Shine-Dalgarno region of *rsbS* during the deletion of *rsbR.* The low levels of RsbS detected in the Δ*rsbR* mutant may be a result of leaky translation due to the ribosome binding at the Shine-Dalgarno sequence for *rsbR,* which was left in the genome when *rsbR* was deleted.

In conclusion, this study has identified a level of redundancy amongst RsbR and its paralogues in the sensing of environmental stress signals. Despite a clear role for SigB during growth in glycerol and sub-lethal hydrogen peroxide, no single paralogue deletion mutant had a significant phenotype, suggesting that no single paralogue is solely responsible for the sensing on an environmental stress signal. Instead, it is probable that each paralogue is capable of sensing an individual element of a particular stress, for example Lmo0799 senses blue light via the cysteine residue at position 56 (Ondrusch and Kreft 2011), and the signals are coordinated within the RsbS:RsbT core. What is unusual, is the highly conserved nature of these proteins despite there being little evidence that they are all required for cell survival. Future work utilising quadruple knock-out mutants, as has been done in *B. subtilis,* would be beneficial in further characterising the individual contributions of RsbR and its paralogues to the response of *L. monocytogenes* to environmental stress sensing (Cabeen *et al.* 2017). Assessing the ability of cells expressing a single RsbR paralogue to respond to environmental stress would further our understanding of the role of the individual paralogues in sensing environmental stress.

5.5 Conclusions

In conclusion, this study builds upon previous investigations into the roles of RsbR and its paralogues as environmental stress sensors. The identification of a previously described secondary mutation in the Δ*rsbR* mutant by WGS

provided evidence that the phenotypes associated with this mutant are likely to be attributable to the secondary mutation. In addition, the quantification of RsbR and RsbS levels by Western blot analysis suggest that the deletion of the RsbS translational start site during the deletion of *rsbR* from the genome reduces the levels of RsbS in this mutant. Finally, the results of the current study suggest that there is a level of redundancy between the RsbR paralogues in stress sensing as the deletion of a single paralogue does not generate a sensitive phenotype to any of the stresses tested in this study. Therefore, it is likely that cells with a single RsbR paralogue deleted are still able to sense and respond to the stresses tested in this study.

Chapter 6: The Impact of Alternative Carbon Sources on SigB Activity and Stress Resistance in *Listeria monocytogenes*

Note: the work in this chapter is part of a joint-first author publication in preparation for submission to *Journal of Food Microbiology* (Crespo Tapia *et al.* 2019). The author contribution included growth analysis, quantitative fluorescence spectroscopy, Western blot analysis and light survival assay conducted at Wageningen University and Research and NUIG, as well as figure and manuscript preparation.

6.1 Abstract

Listeria monocytogenes is an important food-borne pathogen that is ubiquitous in the environment. Common sources of *L. monocytogenes* infections include ready-to-eat food products, including dairy products, seafood and some fruits and vegetables. The ubiquitous nature of *L. monocytogenes* is due to its ability to utilise a variety of carbon sources, and to survive food preservation techniques, usually via the general stress response Sigma factor, SigB. In this study, we investigated the role of SigB in growth in the presence of three common carbon sources, and the ability of these carbon sources to activate SigB. A fluorescent reporter coupled to the promoter of *lmo2230,* a highly SigB-dependent gene, enabled us to measure SigB activity via quantitative fluorescence spectroscopy, Western blot analysis and fluorescence microscopy. Finally, we show how the level of SigB activity induced during food processing alters the sensitivity of the bacterium to common food preservation techniques including heating and acidification. From our study, we conclude that SigB is involved in growth in the presence of glycerol or lactose, and that exposure to lactose induces high levels of SigB activity and resistance to food preservation techniques.

6.2 Introduction

Listeria monocytogenes is a Gram positive bacilli which is found ubiquitously in the environment. Common environmental niches of *L. monocytogenes* include soil, plants, faecal matter and animal fodder (Weis and Seeliger 1975). In the environment, the risk of *L. monocytogenes* infecting humans is low, but its transfer into the food chain, particularly to foods that require no cooking prior to consumption, can result in life-threatening Listeriosis (Farber and Peterkin 1991). To survive in such a variety of niches, ranging from soil to the human gastrointestinal tract, *L. monocytogenes* must be able

to adapt sufficiently to a variety of environmental stresses and available nutrient sources.

In the wide range of environments in which *L. monocytogenes* can survive, the availability of nutrients varies greatly, and therefore *L. monocytogenes* must be able to utilize many different compounds as carbon and/or energy sources. In order to cope with these changing conditions, *L. monocytogenes* has developed a diverse range of transport systems for different sugars (Deutscher *et al.*, 2014). The genome of this bacterium contains a significant amount of ABC transporters and PTS systems, most of which have not been characterised yet.

Common laboratory culture mediums used for the growth of *L. monocytogenes* use glucose or glycerol as the main, if not sole, carbon sources (Amezaga *et al.* 1995, Abram, Su, *et al.* 2008), despite the role of such carbon sources in regulating the expression of genes required for virulence and stress survival having been well studied (Raengpradub *et al.* 2008, Deutscher *et al.* 2014). Studies investigating the effects of glucose metabolism on gene transcription suggest that it is inhibitive of PrfA activity, the main regulator of virulence in *L. monocytogenes* (Deutscher *et al.* 2014). A similar study investigating the effects of glycerol metabolism on PrfA activity suggest that glycerol metabolism activates PrfA, thereby increasing virulence (Joseph *et al.* 2008). Moreover, previous work by Abram *et al.* (2008) has shown that mutants lacking the stress response regulator Sigma B are deficient in glycerol metabolism (Abram *et al.*, 2008). As these studies show, the carbon sources that *L. monocytogenes* is able to metabolise may alter gene transcription and regulation, potentially influencing its ability to survive food processing stresses and infect a human host.

Glucose and glycerol metabolism has been relatively well studied in *L. monocytogenes*, but, as mentioned before, this microorganism has the

potential to be able to metabolise many other compounds as carbon sources. It has been described that *L. monocytogenes* is also able to grow with simple sugars such as mannose and fructose, and more complex molecules like cellobiose and lactose (Abram, Starr, *et al.* 2008). Lactose is a disaccharide composed of one glucose molecule linked to one galactose molecule by a β-1,4-glycosidic bond. Lactose can be found in food-processing environments that *L. monocytogenes* is likely to encounter such as dairy processing environments (McSweeney and Fox 2009).

Previous studies have shown that most *L. monocytogenes* strains are able to utilize lactose as a carbon source to a certain degree (Pine *et al.* 1989), but little is known about the metabolic pathway or the genes involved. It has been reported that *L. monocytogenes* uses only the glucose moiety of the lactose molecule, and exports the galactose moiety out of the cell, but the mechanism by which this happens remains to be elucidated (Pine *et al.* 1989). A more recent paper by (Dalet *et al.* 2003) describes a set of genes that might be involved in the transport of the lactose molecule inside the cell, but the results were not conclusive. The genes of this so called Lpo operon were indeed upregulated during growth in lactose-based media; however, deletion mutants lacking some of the operon elements were still able to grow with lactose (Dalet *et al.* 2003), suggesting that another mechanism might also play a role in lactose metabolism in *L. monocytogenes.*

In *E. coli,* it has been reported that the switch from glucose metabolism to the more complex metabolism of lactose leads to a transitory activation of RpoS, the stress response regulator in *E. coli* (Fischer *et al.* 1998). The impact of lactose metabolism in virulence and/or stress resistance in *L. monocytogenes* has not yet been investigated. The alternative sigma factor, SigB, has been implicated in mediating the general stress response of *L. monocytogenes* (Becker *et al.* 1998). Previous research has reported a role

for SigB in resistance to several stresses associated with food preservation, including acid stress, osmotic stress and temperature stress (Becker *et al.* 1998, Ferreira *et al.* 2001, Sue *et al.* 2004). In addition, *ΔsigB* deletion mutant shows a reduced ability to survive under carbon limiting conditions, suggesting that SigB is involved in resistance to starvation stress (Ferreira *et al.* 2001). The challenge of *L. monocytogenes* cells by acid and heat stress identified a role for carbon starvation in inducing resistance to these stresses (Herbert and Foster 2001), suggesting that nutrient availability can impact the resistance of cells to environmental stresses. A further study by Abram *et al.* (2008), showed a reduced growth rate for the Δ*sigB* mutant in the presence of glycerol. Taken together, the results of these studies suggest that SigB is involved in resistance to environmental stresses, and also the metabolism of some carbon sources.

In this study, we present our findings on the role of SigB in the metabolism of glucose, glycerol and lactose by *L. monocytogenes,* and the impact of the metabolism of these carbon sources on the activation SigB. In addition, we present data highly relevant to the food processing, demonstrating the role of metabolising these carbon sources on the resistance of *L. monocytogenes* to stress treatments commonly utilised in the food processing industry.

6.3 Results

6.3.1 The absence of SigB reduces growth yield in the presence of glycerol or lactose

As the absence of SigB is known to be detrimental during growth in the presence of glycerol in *L. monocytogenes* 10403S (Abram *et al.,* 2008), the growth of *L. monocytogenes* EGDe wild-type and Δ*sigB* deletion mutant strains in the presence of glucose, glycerol or lactose was compared. No

significant differences ($p = >0.05$) were found between the specific growth rates of the Δ*sigB* mutant and isogenic parental strain in any of the carbon sources (Fig 6.1). The growth yield was calculated for each strain after growth in each of the carbon sources, and the Δ*sigB* mutant was found to have a significantly reduced growth yield ($p = \leq 0.05$) compared to the wild-type when grown in the presence of glycerol or lactose. These results suggest that SigB is involved in growth in glycerol and lactose.

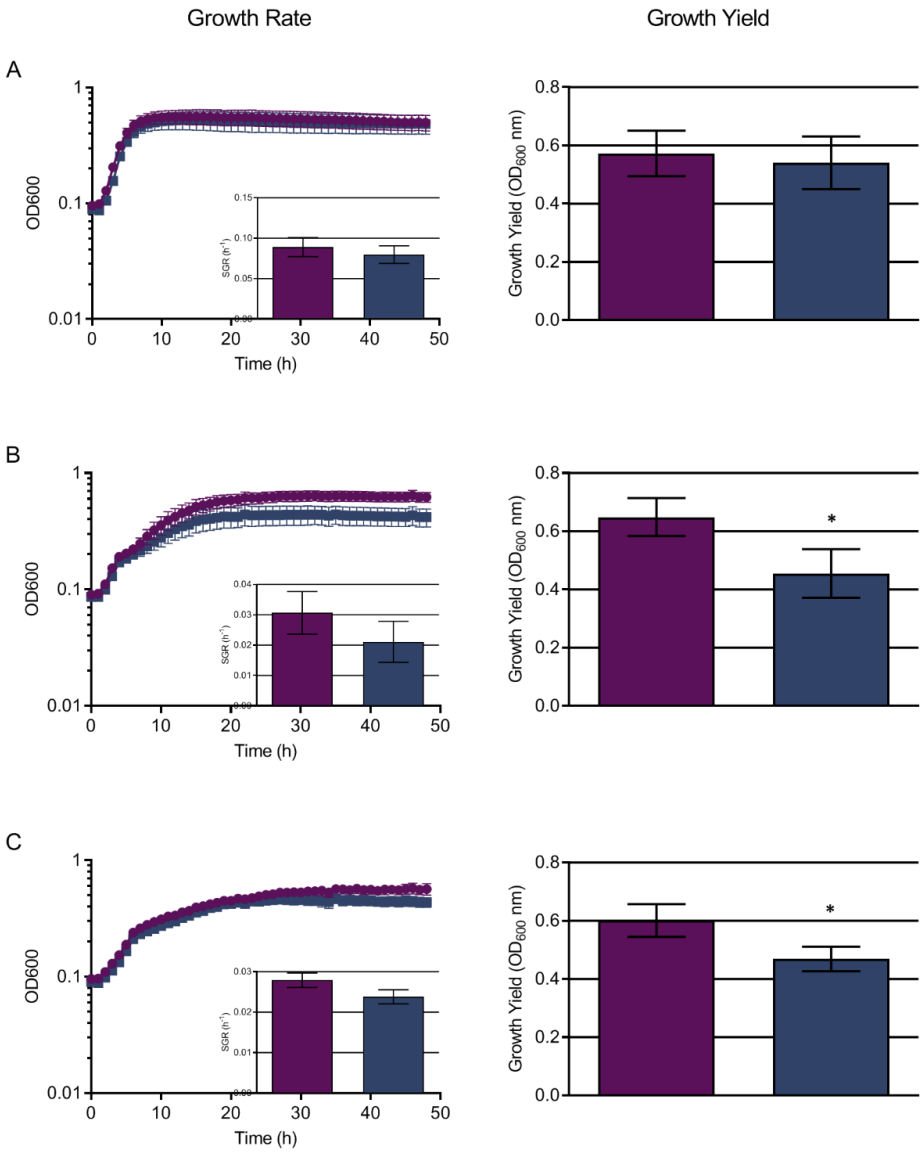

Figure 6. 1 The absence of SigB reduces the growth yield in NB-glycerol and NB-lactose.

EGDe wild-type (purple) and Δ*sigB* deletion mutant (blue) strains were grown in nutrient broth supplemented with either glucose (A), glycerol (B) or lactose (C) (0.4%) at 30°C on a Bioscreen C with continuous shaking. The specific growth rate for both strains was calculated using the exponential growth equation (inset graphs; Wild-Type purple bars, Δ*sigB* deletion mutant tan bars) (Prism 6). Growth yield was calculated using the area under curve analysis (Prism 6). Data are presented as the mean of two biological replicates with SD. (*: P = ≤0.05)

6.3.2 Growth in lactose increases SigB activity

The activation of SigB during *L. monocytogenes* growth with alternative carbon sources was measured. A wild type strain containing a fluorescent eGFP reporter integrated upstream of the SigB-dependent *lmo2230* gene was used (Utratna *et al.* 2012). Through measuring eGFP fluorescence intensity over time, the activation of SigB could be measured. The raw fluorescence intensity values obtained were normalized per OD_{600} to account for differences in cell density between the cultures. Cells grown in the presence of lactose had higher levels of eGFP fluorescence than cells grown in either glucose or glycerol over time (Fig. 6.2A). Overall, cells grown in the presence of glucose exhibited the lowest levels of SigB activation over time (Fig. 6.2A).

To confirm the results obtained from the fluorescent growth curves, Western Blotting using antibodies targeted against the eGFP protein was performed. As expected, the results confirmed those of the fluorescent growth curves, with the highest levels of eGFP detected after growth in the presence of lactose (Fig. 6.2B). The levels of eGFP detected after growth in the presence of glucose or glycerol were not significantly different (Fig. 6.2B), which may be due to the reduced sensitivity of Western blotting compared to quantitative fluorescence spectroscopy.

Fluorescence microscopy pictures of 24-hour cultures grown in the presence of glucose showed a low proportion of fluorescent cells, with low fluorescence intensity (Fig. 6.2C). Cultures grown in the presence of glycerol presented a mixed population of non-fluorescence and fluorescence cells (Fig. 6.2C). Cultures grown in the presence of lactose showed the highest proportion of fluorescent cells, and the highest signal intensity (Fig. 6.2C), confirming the results for the quantitative fluorescence spectroscopy and Western blotting.

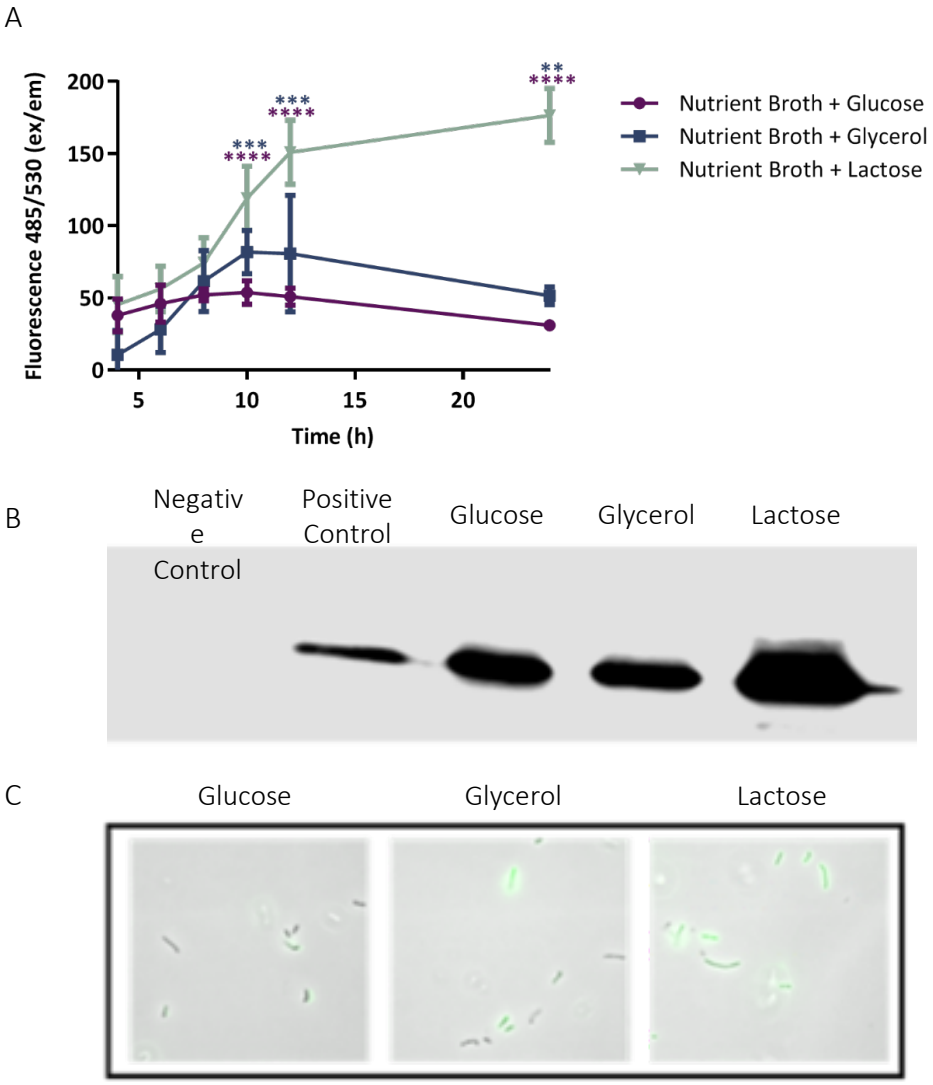

Figure 6. 2 SigB activity is increased during growth in the presence of lactose compared to glucose or glycerol.

EGDe WT integrated pKSV7-p2230-egfp1 cells were grown in nutrient broth supplemented with either glucose, glycerol or lactose (0.4%) at 30°C in flasks with continuous shaking. (A) At specified time points, culture OD_{600} and fluorescence (485/530) (ex/em) were measured. Statistical significance between lactose and either glucose or glycerol was determined at each time point using a two-way ANOVA with Dunnett's multiple comparisons test(*: p = ≤0.05; **: p = ≤0.01; ***: p = ≤0.001; ****: p = ≤0.0001). (B) Protein samples were extracted at 24 h and quantified by Western blotting using anti-eGFP antibodies. EGDe WT cells not carrying the pKSV7-p2230-egfp1 reporter were used as a negative control, and an *E. coli* strain with an integrated pKSV7-p2230-egfp1 plasmid were used as a positive control. (C) Cells were visualised in brightfield and fluorescence microscopy to indicate the proportion of fluorescent cells.

6.3.3 The impact of carbon sources on stress resistance

The role of SigB in resistance to stresses commonly encountered by *L. monocytogenes* in food processing environments has been studied extensively (reviewed in NicAogáin & O'Byrne 2016). To investigate the effect of the exposure of *L. monocytogenes* to alternative carbon sources in food processing environments on tolerance to food preservation techniques, the effect of growth in alternative carbon sources on the sensitivity of *L. monocytogenes* to acid, heat and blue light stress was investigated. Samples were taken after 5 and 24 h of growth, allowing the analysis of both the exponential and stationary phases of growth.

After 20 minutes incubation at 55°C, cells grown in either glucose or glycerol were significantly more sensitive ($p = \leq 0.05$) to the heat compared to those grown in lactose (Fig. 6.3A). After 45 min of incubation at 55°C, the cells grown in lactose showed a 2.5 $\log_{10}$CFU/mL reduction, while cells grown in either glucose and glycerol fell below the limit of detection (100 CFU/mL) (Fig. 6.3A). When the heat inactivation dynamics were performed on cells grown in the presence of alternative carbon sources for 24 hours, the higher resistance of cells grown in lactose became more apparent, with cells showing no significant ($p = \geq 0.05$) decrease in survival after 30 minutes of heat inactivation (Fig. 6.3A). This is in contrast to the cells grown in glucose or glycerol, which showed a significant ($p = \leq 0.05$) decrease in survival compared to the lactose grown cells after just 20 min (Fig. 6.3A). After 45 minutes of heat inactivation, cells grown in the presence of lactose showed a 1 $\log_{10}$CFU/mL reduction from the initial cell count (Fig 6.3A), while cells grown in the presence of glycerol showed a 4 $\log_{10}$CFU/mL reduction and cell counts for cells grown in the presence of glucose fell were below the limit of detection (Fig. 6.3A).

The acid inactivation results followed a similar trend to the heat treatment (Fig. 6.3B). Cultures incubated for 5 h were treated with NB acidified to pH 3, while 24 h cultures were treated with NB acidified to pH 2. This difference in pH was to account for cells cultured for 24 h showing increased resistance to acid stress, irrespective of carbon source, compared to those cultured for 5 h (data not shown.

The pH inactivation dynamics of cells grown for 5 h showed that growth in the presence of lactose induced higher resistance to low pH, with cell counts significantly ($p = \leq 0.05$) higher after 40 min incubation at pH 3 compared to those for cells grown in glucose or glycerol (Fig. 6.3B). Cells cultured for 24 h in the presence of lactose were significantly ($p = \leq 0.05$) more resistant to acid stress after 15 min, 30 min and 60 min of incubation at pH 2 (Fig. 6.3B). After 75 min of incubation, there was no significant ($p = \geq 0.05$) difference in the resistance between any of the cultures, potentially due to the adaptation of *L. monocytogenes* cells to acid stress (O 'Driscoll *et al.* 1996).

A previously published study has shown a requirement for SigB in blue light resistance at 30°C (O'Donoghue *et al.,* 2016). In contradiction to the acid and heat stress assays, cells grown in lactose were significantly ($p = >0.05$) more sensitive to 35mW cm^2 blue light than those grown in glucose or glycerol(Fig. 6.3C). However, in agreement with the acid and heat stress assays, cells grown for 24 h in the presence of lactose were significantly more resistant ($p = \leq 0.05$) to killing by blue light compared to cells grown in the presence of either glucose or lactose. Taken together, these results suggest that prolonged exposure to lactose may induce resistance of *L. monocytogenes* to killing by blue light, possibly due to the activation of SigB.

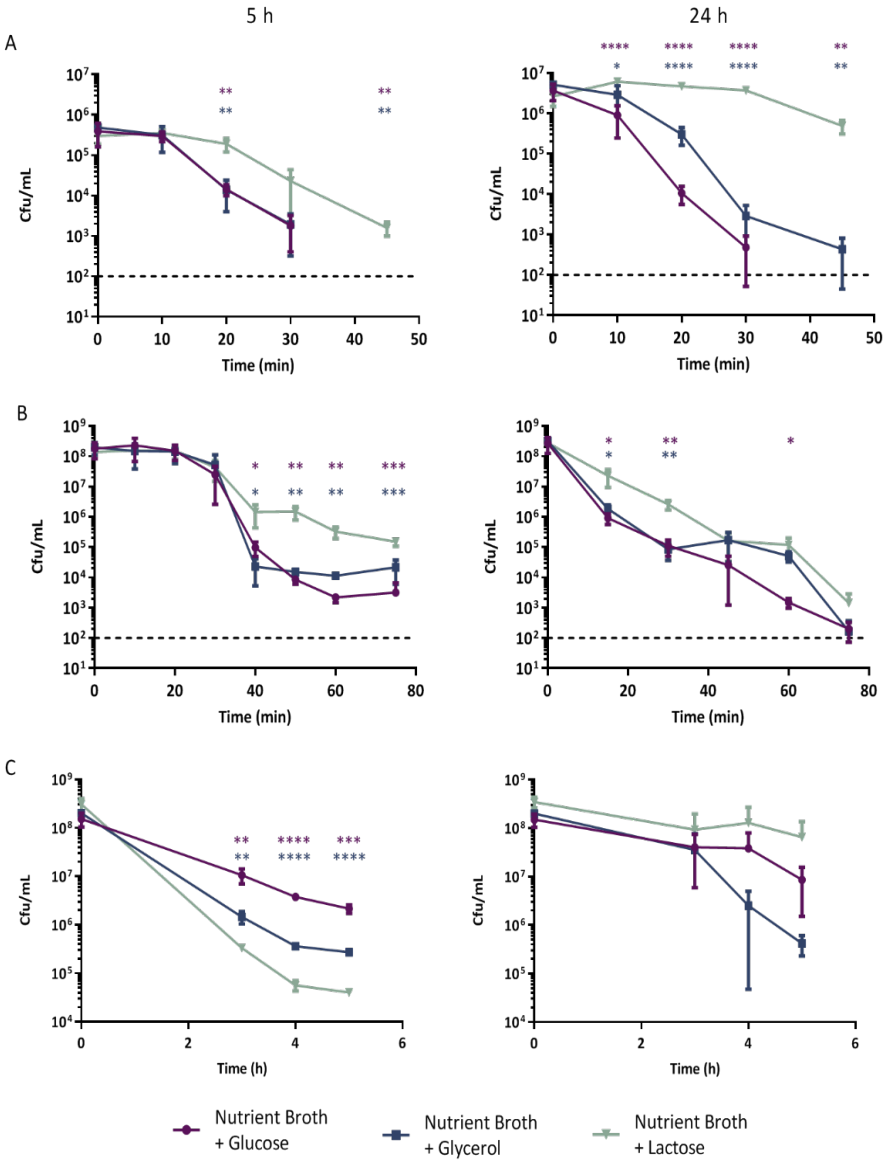

Figure 6. 3 Resistance of *L. monocytogenes* to acid, heat and visible light stress is significantly higher in cells grown in lactose compared to glucose or glycerol.

Cells were collected after 5 h and 24 h growth and their resistance to (A) heat, (55°C) (B) acid (pH 3 and pH2), and (C) visible light (35 mW cm^2) stress was measured over time. Each point represents the average cell count of two biological replicates, and error bars show the standard deviation of the two biological replicates. Statistical significance between lactose and either glucose or glycerol was determined at each time point using a two-way ANOVA with Dunnett's multiple comparisons test(*: p = ≤0.05; **: p = ≤0.01; ***: p = ≤0.001; ****: p = ≤0.0001). Limit of detection indicated by dashed line.

6.4 Discussion

6.4.1 SigB plays a role in the growth of *L. monocytogenes* in glycerol and lactose

In the current study, a potential role for SigB during growth in the presence of glucose, glycerol and lactose was identified, with the Δ*sigB* mutant unable to produce the same growth yield as the wild-type during growth in glycerol and lactose. Previous investigations into the role of SigB in the metabolism of *L. monocytogenes* identified a role for SigB in glycerol metabolism in *L. monocytogenes* 10403S, with the Δ*sigB* mutant showing a significantly reduced specific growth rate compared to the wild-type (Abram *et al.* 2008). The current study did not identify a significant alteration in growth rate between the wild-type and Δ*sigB* mutant strains (Fig. 6.1), however the Δ*sigB* mutant showed a significant reduction in growth yield when grown in either glycerol or lactose. Proteomic approaches employed by Abram *et al.* (2008), identified several genes hypothesised to be involved in the metabolism of glycerol as SigB-dependent, therefore suggesting that the Δ*sigB* mutant is unable to produce the proteins required for glycerol metabolism.

6.4.2 *L. monocytogenes* activates SigB during growth in lactose

To gain more insight into the role of SigB during growth in glycerol and lactose, several assays quantifying the levels of SigB activity during growth in glucose, glycerol and lactose were performed. The results showed that SigB activity, estimated by the quantification of a strongly SigB-dependent protein, is lowest during growth in the presence of glucose, and highest during growth in the presence of lactose (Fig. 6.2). In line with these findings, previous work by Wang et al (2014) has shown that the metabolism

of glucose, or other PTS sugars like cellobiose, have a negative impact in the transcription levels of SigB–related genes. In contrast, previous work in E. coli has shown that, when cells are switched from glucose to lactose, RpoS suffers a temporary activation while the cells adjust to the new, more complex, carbohydrate (Fischer *et al.* 1998). This is a common bacterial behaviour known as the starvation-stress response, in which bacteria react to a sudden lack of a preferred nutrient in the media as a stress signal, and their stress mechanisms are induced.

While this study was able to identify a mechanism through which SigB enables growth in the presence of lactose, we hypothesize that *L. monocytogenes* interprets either the presence of lactose or its by-products, as a situation of stress. It is the interpretation of this signal that results in the activation of SigB, and the expression of stress-related genes, as opposed to a requirement for the expression of these genes in lactose metabolism. Previously, the absence of SigB has been shown to induce a growth advantage in conditions containing sub-lethal stress (Abram *et al.* 2008), so it would be plausible to expect the Δ*sigB* mutant to display a growth advantage compared to the wild-type during growth in the presence of lactose if there was no role for SigB in lactose metabolism. Further work investigating the mechanism of lactose metabolism in *L. monocytogenes* is required before either of these hypotheses can be further considered.

An alternative hypothesis for the reduced growth yield and increased SigB activity in lactose considers the structure of the molecule itself. Lactose is a complex carbohydrate composed of a glucose and a galactose moiety. As previously described, *L. monocytogenes* utilises only the glucose moiety for growth, and the galactose moiety is exported from the cell via an unknown mechanism (Pine *et al.* 1989). Both an increased intracellular or extracellular concentration of galactose may be sensed by the cell as a toxicity signal, and

trigger the stress response, but this theory needs to be investigated in future work.

6.4.3 Growth in the presence of lactose may lead to the resistance of *L. monocytogenes* to food preservation techniques

In order to investigate the potential of alternative carbon sources to induce resistance to commonly utilised food preservation techniques via activation of the general stress response in *L. monocytogenes*, several stress treatments were performed. When heat and acid resistance was investigated, a positive correlation between resistance to these stresses and increasing SigB activity was shown through cultivation with lactose (Fig. 6.3A and B). This result matches previously published studies demonstrating a role for SigB in resistance to these stresses (Ferreira *et al.* 2001).

Blue light killing has been hypothesized to be via the generation of ROS (O'Donoghue *et al.* 2016). Previous literature suggests that resistance of *L. monocytogenes* to ROS is not mediated by SigB (Ferreira *et al.* 2001), and, conversely, the Δ*sigB* mutant has been shown to survive exposure to ROS significantly more than the wild-type (Boura *et al.* 2016). The current study found that exponentially growing cells grown in the presence of glucose exhibited the highest levels of resistance to blue light stress (Fig. 6.3C), suggesting that lower levels of SigB activity are beneficial for resistance to this stress in the exponential phase of growth. Paradoxically, the stationary phase cells grown in the presence of lactose, and showing the highest levels of SigB activity, showed the highest levels of resistance to blue light (Fig. 6.3C). Taken together, these results suggest that carbon sources do alter the resistance of *L. monocytogenes* to blue light as they do for heat and acid stress, however the growth phase of the cells also influences their susceptibility to killing by blue light, as shown in Fig. 3.1.

6.5 Conclusions

In this study, we have highlighted a potential role for SigB in the growth of *L. monocytogenes* in the presence of glycerol and lactose, but not in glucose. Via assays measuring the levels of SigB activity, we have gone on to show that not only is the Δ*sigB* mutant inhibited during growth in the presence of these carbon sources, but SigB activity is higher during growth in the presence of either glycerol or lactose than it is in glucose. Finally, this study has identified a novel role for carbon sources in influencing the resistance of *L. monocytogenes* to commonly utilised methods of bacterial inactivation in the food processing industry, including acid and heat stress.

Chapter 7: Discussion

7.1 Overview

L. monocytogenes is an important foodborne pathogen that is ubiquitous in the environment, where it will encounter a variety of environmental stresses including blue light. In the course of this study, the relationship between *L. monocytogenes* and blue light has been investigated. The first part of this study investigated the influence of environmental factors on the sensitivity of *L. monocytogenes* to killing by blue light (Chapter 3), with the results from these investigations determining the conditions at which whole transcriptome response of *L. monocytogenes* to blue light exposure was investigated (Chapter 4). The second part of the study focused upon the roles of RsbR and its paralogues as environmental stress sensors (Chapter 5), and the role of SigB activation by alternative carbon sources in conferring resistance to potential food processing industry sanitising agents (Chapter 6). The results of this study have yielded important considerations for implementing blue light as a sanitising agent in the food processing industry, and have built upon current knowledge to further characterise the role of SigB in protecting *L. monocytogenes* from the oxidative damage caused by blue light.

7.2 Environmental factors influence the resistance of *L. monocytogenes* to blue light

While it is an important pathogen, *L. monocytogenes* is able to reside within the environment where it will encounter a variety of stresses, including light exposure. Within the host, it is unlikely that *L. monocytogenes* will encounter light exposure, and previous research has demonstrated that the chromophore stability of Lmo0799 decreases at temperatures above 30°C (Chan *et al.* 2013). For this reason, the impact of growth temperature on

the sensitivity to and sensing of blue light by *L. monocytogenes* was investigated.

The phenomenon of cross-resistance in *L. monocytogenes* between environmental stresses has been previously reported (Lou and Yousef 1997), and so it was not unexpected that changes in the growth phase or growth temperature would influence the susceptibility of *L. monocytogenes* to blue light (Figure 3.1). This study has predicted that altered resistance of *L. monocytogenes* to blue light is under control of SigB, as the resistance of the Δ*sigB* mutant to blue light was unaffected by changes in growth phase or temperature (Figure 3.1). Previous investigations into the transcription of *sigB* and SigB-dependent genes in response to osmotic stress have shown a temperature-dependent effect, with increased salt concentrations required at 7°C compared to 37°C to maximise SigB activity (Bergholz *et al.* 2012), however this is likely due to increased basal SigB activity at 7°C compared to 37°C during the presence of cold stress. Research investigating the effects of growth temperature on resistance to H_2O_2 has shown an increase in the resistance of *L. monocytogenes* to H_2O_2 as temperature decreases (Ochiai *et al.* 2017), possibly due to an increase in SigB activity as temperature decreases. However, in contrast to these findings, Boura *et al.* (2016) has shown that the absence of SigB is beneficial for resistance to H_2O_2, independent of growth temperature. Together, these results suggest that there is a complex regulation of stress responses in *L. monocytogenes*, with the role of SigB in these responses potentially influenced by a variety of factors.

The current study presents evidence of a complex inter-regulatory network between growth phase, growth temperature and SigB. When the effect of growth phase on blue light sensitivity of *L. monocytogenes* at 37°C was

investigated, wild-type cells at exponential phase were significantly more sensitive than cells at stationary phase, while the Δ*sigB* mutant was unaffected by the change in growth phase (Figure 3.1A and B). When considering the effect of growth temperature on the resistance of stationary phase cells to blue light, the results of the current study suggest that SigB is required for resistance to blue light at 30°C, but not at 37°C (Figure 3.1C). SigB activity is known to be higher in stationary phase *L. monocytogenes* cells at 30°C compared to 37°C (O'Donoghue 2017), providing further evidence that SigB contributes to resistance to blue light under these conditions. The temperature-dependency of blue light sensing may suggest that it plays an important role in protecting *L. monocytogenes* from oxidative damage caused by sunlight exposure during inhabitation of the soil.

Global gene transcription data for the entire *L. monocytogenes* genome identified several genes that show significantly altered, either increased or decreased, transcription at both 30°C compared to 37°C and at stationary phase compared to exponential phase in BHI (Toledo-Arana *et al.* 2009, Supplementary Table 6), which are listed in Table 7.1. From these data it is possible to construct a hypothetical model to suggest the possible roles these genes may play in the increased sensitivity of *L. monocytogenes* to blue light at 37°C in exponential phase.

Table 7. 1. Genes with significantly altered expression at 30°C compared to 37°C and at stationary phase compared to exponential phase.

Gene	Transcription at 30°C Compared to 37°C[1]	Transcription at Stationary Phase Compared to Exponential Phase[2]	Encoded Protein Function
lmo0367	Downregulated	Downregulated	Ferrous iron transport peroxidase EfeB
lmo0484 isdG	Downregulated	Downregulated	Heme degrading monooxygenase IsdG
lmo1007	Downregulated	Downregulated	Unknown
lmo1945	Upregulated	Upregulated	Substrate-specific component RibU of riboflavin ECF transporter
lmo1961	Downregulated	Downregulated	Thioredoxin reductase; similar to oxidoreductases and likely to be involved in redox regulation
lmo2183	Downregulated	Downregulated	Heme transporter IsdDEF, permease component IsdF
lmo2184	Downregulated	Downregulated	Heme transporter IsdDEF, lipoprotein IsdE
lmo2185	Downregulated	Downregulated	Cell surface protein IsdA, transfers heme from hemoglobin to apo-IsdC
lmo2186	Downregulated	Downregulated	NPQTN cell wall anchored protein IsdC
lmo2467	Upregulated	Upregulated	Chitin binding protein
lmo2522	Downregulated	Downregulated	Cell-wall binding protein

[1]Experiment conducted in BHI broth in exponential phase.

[2]Experiment conducted in BHI broth at 37°C.

[ŧ]Data obtained from study by Toledo-Arana *et al.* (2009).

The increased transcription of *lmo2183-2186,* encoded within Operon 396 (Bécavin *et al.* 2017) at 37°C is likely to result in an increase in intracellular haem due to an increase in haem transporters. High levels of intracellular

haem have been associated with reactive oxygen species (Ascenzi *et al.* 2005) as haem acts as a photosensitiser, which may exacerbate the effects of blue light. While these theories are very speculative and further experiments are required to provide evidence for them, a previous study has found that the co-incubation of *L. monocytogenes* with 5-aminolevulinic acid (ALA), a compound which is converted to porphyrins via the haem biosynthetic pathway, increases the bactericidal effects of blue light (Buchovec *et al.* 2010, Vollmerhausen *et al.* 2017). In addition, growing *L. monocytogenes* in the presence of hemin, ferric chloride haem, also increases the sensitivity of *L. monocytogenes* to blue light (Patricia Dos Santos, personal communications). The deleterious role of SigB in this reaction may arise from the SigB-dependent expression of *lmo0484* (Toledo-Arana *et al.* 2009), which encodes a haem degrading monooxygenase, IsdG. IsgD is required to convert haem to biliverdin-IX-A, Fe^{2+}, CO and NADPH-hemoprotein reductase (The Uniprot Consortium, 2019). NADPH-hemoprotein reductase is a photosensitiser, generating reactive oxygen species in the presence of light. In the absence of SigB, *lmo0484* is not expressed, and therefore the breakdown of haem cannot occur, preventing the production of biliverdin-IX-A, Fe^{2+}, CO and NADPH-hemoprotein reductase.

Secondly, in the transcriptomic data provided by Toledo-Arana et al (2009), *lmo0367* was downregulated at both 30°C and stationary phase (Table 7.1). A BLAST protein search using the amino acid sequence of Lmo0367 identified 51.8% homology with *B. subtilis* EfeB. EfeB has been reported to be required for the oxidation of Fe^{2+} to Fe^{3+} (Miethke *et al.* 2013). In addition, EfeB is required to reduce the hydrogen peroxide which accumulates in the cell with increasing iron concentrations, and uses the oxygen molecule generated in this reaction to oxidise the Fe^{2+} produced during the breakdown of haem to Fe^{3+} (Miethke *et al.* 2013). Therefore, this

creates a paradox in *L. monocytogenes* as the transcriptional data of Toledo-Arana et al (2009) suggests that *lmo0367* is upregulated at 37°C, and therefore might be expected to confer resistance to blue light via the breakdown of reactive oxygen species. However, the Fet3p ferroxidase in yeast, which also oxidises Fe^{2+} to Fe^{3+}, plays no role in the breakdown of hydrogen peroxide and instead uses O_2 as an electron donor for the reaction (Stearman *et al.* 1996, Miethke *et al.* 2013). Therefore, we can speculate that the increased transcription of *lmo0367* at 37°C may lead to an increase in O_2^- , a type of reactive oxygen species, as a result of the oxidation of Fe^{2+} to Fe^{3+}, and may account for the increased sensitivity of cells grown at 37°C to blue light compared to those grown at 30°C. The model described is summarised in Figure 7.1.

The hypothesis that increased haem transport contributes to the increased sensitivity of *L. monocytogenes* to blue light at 37°C in exponential phase will require further investigation. It could be tested by using both deletion mutants that alter either haem transport or metabolism, and strains with the same genes encoded on a plasmid with a selectively inducible promoter, for example the IPTG-induced promoter that has previously been used in *L. monocytogenes* to investigate the requirement of LLO in virulence (Dancz *et al.* 2002). An increased resistance of the deletion mutants to blue light, paired with an increased sensitivity of cells containing the same gene to blue light after expression has been induced, would suggest that haem biosynthesis may be involved in the increased sensitivity of *L. monocytogenes* to blue light under certain growth conditions. However, it would be best to measure both the intracellular and extracellular levels of haem to confirm that uptake of haem is required for it to act as a photosensitiser.

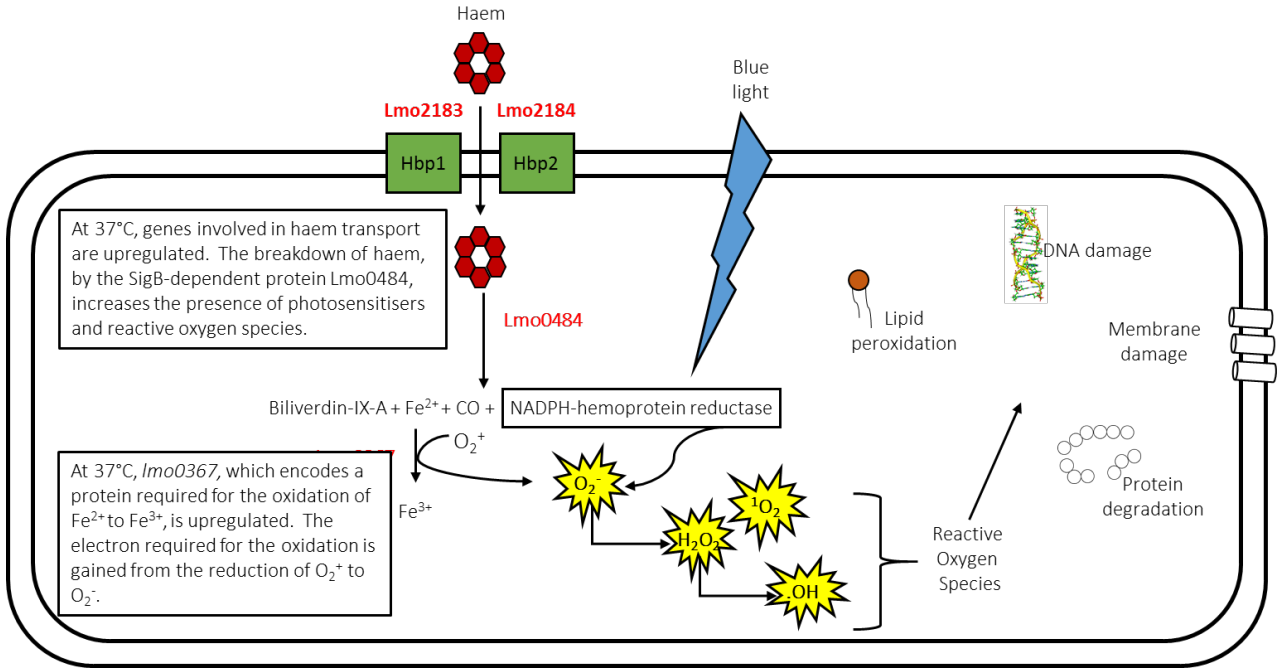

Figure 7. 1 The increased sensitivity of *L. monocytogenes* to blue light in exponential phase at 37°C may be due to increased haem transport.

Both when cells are grown at 37°C and when they are at exponential phase, transcription of both *lmo2183* and *lmo2184,* encoding Haem-binding proteins 1 and 2 (Hbp1 & 2),respectively, is upregulated, potentially increasing intracellular haem concentrations. Intracellular haem is broken down by Lmo0484, which has been shown to be SigB-dependent, to form biliverdin-IX-A, Fe^{2+}, CO and NADPH-hemoprotein reductase. Upon excitation with blue light, NADPH-hemoprotein reductase acts as a photosensitiser and reacts to generate reactive oxygen species responsible for cell damage. In addition, Fe^{2+} can be oxidised to Fe^{3+} in the presence of Lmo0367, encoded by *lmo0367* whose transcription is upregulated at 37°C and in exponential phase, using molecular oxygen as an electron donor. Singlet oxygen is a by-product of this reaction and is also a form of reactive oxygen species.

The ability of ethanol to increase the resistance of *L. monocytogenes* to blue light via a mechanism that is independent of SigB and CesRK, and at least partially independent of LisRK (Figures 3.4 and 3.5) was another unexpected, but important, finding of the current study. Ethanol is a common sanitising agent used in the food processing industry (Gravesen *et al.* 2005), and therefore *L. monocytogenes* is likely to come into contact with both ethanol and blue light should blue light be developed as a sanitising agent. With the exception of the LisRK and CesRK two-component systems (Cotter *et al.* 1999, 2002, Kallipolitis *et al.* 2003, Gottschalk *et al.* 2008), the description of genes and proteins potentially involved in the response of *L. monocytogenes* to ethanol stress is limited.

In *E. coli,* ethanol exposure leads to a breakdown of the cell wall and a reduction in membrane integrity, of which a by-product is reactive oxygen species (Cao *et al.* 2017). *Staphylococcus aureus* has been shown to upregulate a selection of general stress response and heat shock proteins in response to ethanol exposure (Pando *et al.* 2017), which may be beneficial in resisting damage by ROS. Together these data suggest that the exposure of *L. monocytogenes* to ethanol may induce a response to reactive oxygen species generated through cell wall degradation, in effect priming the cell for the reactive oxygen species encountered when exposed to blue light. While we have shown a requirement for SigB in blue light resistance under certain conditions in the current study (Figure 3.1), it is unlikely that ethanol generates resistance to blue light via the induction of a general stress response, as the protective mechanism induced by ethanol is SigB-independent (Figure 3.4).

Additional work in *E. coli* has shown that ethanol exposure can inhibit transcription and translation through effects on RNA polymerase (Haft *et al.* 2014), possibly suggesting that the inhibition of *L. monocytogenes*

transcription and translation leads to a resistance to blue light. A preliminary investigation into whether ethanol confers resistance to blue light via *de novo* protein synthesis, showed that the addition of chloramphenicol alone, which arrests protein translation, is able to induce a significant level of resistance to blue light (Appendix 1). Therefore, ethanol may induce resistance to blue light partially through the inhibition of *de novo* protein synthesis.

While the reason the inhibition of *de novo* protein synthesis conferring resistance to blue light is not yet understood, it is possible that it is due to energy conservation. There is a finite supply of energy within a cell, and so a decrease in protein synthesis would result in an incease in energy that is available to be used for cell protection and repair mechanisms. An alternative hypothesis may be due to the arrest in cell growth as a result of the inhibition of *de novo* protein synthesis. If the cell is not actively growing, there will be a reduction in the number of potential targets for reactive oxygen species to damage e.g. ssDNA and mRNA.

Finally, when the role of carbon sources on resistance to blue light was examined, cells grown to exponential phase, but not stationary phase, the presence of lactose in the medium resulted in an increased sensitivity of the cells to blue light compared to when cells were cultured in the presence of glucose (Fig. 6.3C). Cells grown in the presence of lactose showed higher levels of SigB activity compared to cells grown in glucose (Fig. 6.2), suggesting that there is a negative correlation between resistance to blue light and SigB activity at 30°C (Fig. 6.3C). The comparison of SigB activity between the exponential and stationary phase of growth in the absence of additional environmental stress indicates that SigB activity is higher in stationary phase (Utratna *et al.* 2011, 2012). This is highly likely due a

decrease in nutrient availability and build-up of metabolic waste products in the environment during stationary phase.

7.3 Blue light exposure alters gene transcription in *L. monocytogenes*

In previous investigations on the molecular response of *L. monocytogenes* to blue light, RT-PCR or Northern blot analysis targeted SigB-dependent genes (Ondrusch and Kreft 2011) to determine whether SigB was activated in response to light. While these techniques enabled the role of SigB in the response of *L. monocytogenes* to blue light to be investigated, other transcriptomic changes were not studied. Through the use of RNA seq analysis, whole genome transcriptomic changes were measured in response to blue light exposure (Chapter 4). In addition, the inclusion of the Δ*sigB* and Lmo0799 C56A mutants in this analysis enabled the roles of these two proteins in this response to be assessed (Chapter 4).

Having established that SigB is required for the resistance of *L. monocytogenes* to blue light at 30°C (Chapter 3), the question of whether genes outside of the SigB regulon showed changes in transcription to blue light remained. Although the Δ*sigB* mutant showed a significant reduction in survival in the presence of blue light compared to the wild-type, it was not completely killed by blue light during the course of the assay (Fig. 4.1). The Δ*sigB* mutant showed a ~98% reduction in transcriptomic changes in response to blue light compared to the wild-type (Fig. 4.4), altering the expression of only 10 genes in response to blue light exposure compared to the 603 altered in the wild-type, suggesting the response of *L. monocytogenes* to blue light stress is highly SigB-dependent at this dose. A previous study by Young et al (2013) generated results suggesting that the specificity of the response of *B. subtilis* to a stress is dependent upon the

rate at which the bacteria encounter the stress. The study found that slow exposure to either salt or ethanol stress resulted in the upregulation of individual stress genes, whereas fast exposure to the same stresses resulted in the upregulation of individual stress genes and genes of the general stress response (Young *et al.* 2013). Based on the ability of *L. monocytogenes* to grow in light intensities as high as 2 mW cm^2 (O'Donoghue *et al.* 2016), it can be assumed that the 0.6 mW cm^2 used in the current study (Chapter 4) would represent a low exposure to blue light stress, and therefore it is possible that exposing the cells to higher light intensities would have resulted in additional transcriptomic changes, including changes that are independent of SigB. However, future work investigating whole genome transcriptional changes in response to both lower and higher light intensities may be useful in identifying more specific genes involved in the response of *L. monocytogenes* to blue light.

The Δ*sigB* mutant showed significant decreases in the transcription of only two genes in response to blue light exposure that were also significantly affected in the wild-type (Fig. 4.4). One of these genes, *lmo2346*, encodes an amino acid ABC transporter and is downregulated during growth in glycerol (Joseph *et al.* 2008). The second, *lmo2343,* shows homology to the *ytnJ* gene of *B. subtilis* which encodes part of the YtmI operon involved in the metabolism of sulphur (Burguière *et al.* 2005). While these two genes are co-transcribed in Operon 419 (Bécavin *et al.* 2017), they were the only genes in the operon identified in this study to show a significant change in expression. In *L. monocytogenes,* Lmo2343 is hypothesised to be involved in L-cysteine transport, and transcription of *lmo2343* is repressed by AgrA (Garmyn *et al.* 2012). An interesting study noted that the combination of L-cysteine (2%) solution and intense light pulses resulted in a significant increase in the killing of *L. innocua* on avocado, compared to intense light pulses alone (Ramos-Villarroel et al, 2011), suggesting that L-cysteine may

act as a photosensitiser. Therefore, *L. monocytogenes* may decrease the transcription of *lmo2343* in the presence of blue light to reduce the uptake of cysteine and reduce the presence of intracellular photosensitisers that may increase the sensitivity of the cell to blue light.

As the current study demonstrated that ethanol induces resistance to blue light (Chapter 3), it was interesting to examine the transcriptomics data for effects that might suggest a mechanism for this response. Consistent with the adaptation assay results (Fig. 3.5), none of the genes encoding either the LisRK or CesRK two-component systems showed altered transcription in the presence of blue light (Fig. 4.2). This suggests that neither or these systems is likely to be involved in the response to ethanol exposure that induces resistance to blue light. Furthermore, no significant changes in the transcription of *resD, phoP, lmo1745,* or *degU,* genes previously identified to be involved in the response of *L. monocytogenes* to ethanol (Williams *et al.* 2005), were identified (Fig. 4.2), suggesting that the proteins encoded by these genes are unlikely to be involved in the cross-protective response of ethanol and blue light. Interestingly, two genes encoding alcohol dehydrogenase enzymes, *lmo0613,* and *lmo2573,* were significantly upregulated in the presence of blue light (Fig. 4.2), indicating that they may be involved in conferring resistance of *L. monocytogenes* to blue light. However, both genes were downregulated in the Δ*sigB* mutant, suggesting that, if they are involved in the cross-protective response between ethanol and blue light, they are not the only genes involved, as this response is independent of SigB (Fig. 3.4). It is important to note that this study only investigated the ability of ethanol exposure to induce cross-resistance to blue light and not the ability of blue light to induce cross-protection to ethanol. Therefore, it is unlikely that the genes induced by blue light are the same genes induced by ethanol which confer the protective effect.

7.4 Loss of Lmo0799 Cys56 leads to an intermediate change in gene transcription in response to blue light

A mutation in Lmo0799 changing Cys56 to Ala56 generates a light blind mutant, but does not alter the resistance of *L. monocytogenes* to blue light (Fig. 4.1). This was surprising as it has previously been shown that removal of this cysteine residue inhibits the ability of *L. monocytogenes* to respond to blue light (Ondrusch and Kreft 2011, Chan *et al.* 2013, Tiensuu *et al.* 2013, O'Donoghue *et al.* 2016), therefore it would not be unreasonable to assume that *L. monocytogenes* would be more sensitive to killing by blue light if it was unable to sense and respond to it. In order to determine whether this was due to the *lmo0799* C56A mutant activating SigB via an alternative mechanism, the transcriptomic response of this mutant in response to blue light was compared to that of the wild-type and the Δ*sigB* mutant (Fig. 4.6). The results of this analysis suggest that the *lmo0799* C56A mutant is able to activate SigB in response to blue light, however the level of activation is lower than that shown for the wild-type. When the results of the RNA seq analysis were checked by RT-PCR, the Lmo0799 C56A mutant did not show a significant increase in the transcription of *sigB, lmo2230* or *opuCA* over time. This result suggests that, at least in the absence of of a functional light sensor, *L. monocytogenes* is able to activate SigB in response to blue light via an alternative mechanism.

A study by Yee *et al.* (2015) demonstrated that, in the absence of the conserved cysteine residue, LOV domains are able to reduce the FMN to the neutral semiquinone (NSQ) state in the presence of light photons, which is able to modulate downstream signalling in a way that is equivalent to that of cysteine adduct formation. In a LOV domain with the conserved cysteine residue present, blue light excites the flavin, leading to the formation of a covalent adduct between the flavin ring, C4a specifically, and the cysteine

residue (Yee *et al.* 2015). As a result of this, the N5 atom of the flavin gains a proton, causing the amide side chain of a conserved glutamine residue to turn 180° (Yee *et al.* 2015). The turning of the amide side chain leads to alterations in hydrogen bonding throughout the α/β PAS domain of the LOV domain, and ultimately to changes in signalling responses (Yee *et al.* 2015). In the absence of the conserved cysteine residue, it is possible that the flavin is also reduced to the NSQ state, in which N5 is also protonated (Yee *et al.* 2015). As the N5 is also protonated, the same changes in hydrogen bonding could still occur, leading to the same changes in signalling responses (Yee *et al.* 2015) (Fig. 7.1). This suggests that *L. monocytogenes* may still be able activate SigB in response to blue light when Cys56 has been mutated to Ala. This offers a potential explanation for why the Lmo0799 C56A mutant does not have an increased sensitivity to blue light, and why the mutant is also able to partially activate SigB in response to blue light.

As highlighted in a follow on study by Kopka *et al.* (2017), the study by Yee *et al.* (2015) uses high light intensities in order to induce reduction of the flavin to the NSQ state (30 mW cm^2 and 50 mW cm^2). The present study used 35 mW cm^2 to assess the resistance of *L. monocytogenes* wild-type and Lmo0799 C56A mutant to killing by blue light (Fig. 4.1), but only 0.6 mW cm^2 to assess the effect of blue light on the transcriptomic response (Fig. 4.6). Therefore, it seems possible that in the presence of lethal light intensities, full activation of SigB could occur in the absence of Lmo0799 Cys56 via the reduction of the flavin to the NSQ state. This might help explain the absence of a light-sensitivie phenotype for the *lmo0799* C56A mutant. However, there is a possibility that at lower light intensities e.g. <1 mW cm^2, there is insufficient photoexcitation of the flavin to reduce it to the NSQ state. In this case, it is likely that *L. monocytogenes* activates SigB either via an alternative mechanism, as it can for cold stress in the absence of RsbV (Utratna *et al.* 2014), or in response to a secondary stress associated with

blue light. Future work should seek to determine whether there is a minimum light intensity required for sufficient photoexcitation of the flavin to convert it to the NSQ state. If it is found that 0.6 mW cm^2 is insufficient for conversion to the NSQ state, investigations into the possibility of SigB activation via an alternative mechanism or in response to a secondary stress should be conducted.

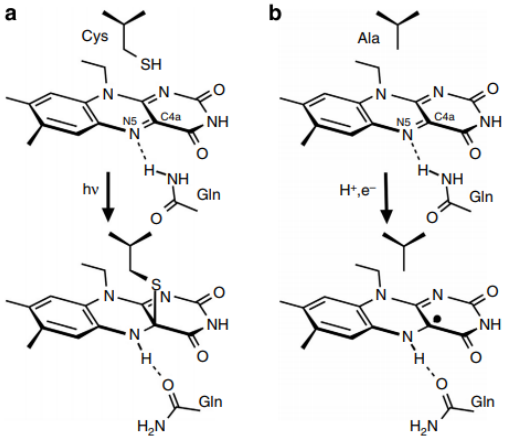

Figure 7. 2. The effect of blue light photons on the flavin in the presence and absence of the conserved cysteine residue in Lmo0799.

(A) In the presence of the conserved cysteine residue, photoexcitation of the flavin leads to the formation of a flavin mononucleotide adduct at C4a between the flavin and the cysteine. As a result of this, a proton is added to N5 and the amide side chain of the glutamine residue is turned 180°. This turn in the amide side chain ultimately results in changes in downstream signalling. (B) In the absence of the conserved cysteine residue, photoexcitation of the flavin results in its reduction to the neutral semiquinolone (NSQ) state. As a result of this, N5 becomes protonated and events can proceed in the same way that they do in the presence of the conserved cysteine. Figure adapted from Yee et al (2015).

7.5 Redundancy exists between the RsbR paralogues of the stressosome in their role as stress sensors

The role of RsbR and each of its paralogues in stress sensing was extensively investigated through a variety of stress tolerance assays (Chapter 5). Building upon previous work by O'Donoghue (2017), and also on work in the current study showing a role for SigB in the utilisation of glycerol and lactose

(Chapter 6), it was expected that certain deletion mutants devoid of a single RsbR paralogue would exhibit a similar stress sensitive phenotype as the Δ*sigB* mutant. It was therefore unexpected that none of the mutants exhibited a stress sensitive phenotype similar to that of the Δ*sigB* mutant in any of the assays investigated in this study (Chapter 5).

However, research into the roles of RsbR and its paralogues in the sensing of ethanol stress in *B. subtilis,* indicated that all of the RsbR paralogues were able to detect and respond to ethanol stress, but each paralogue coordinated a response that differed in length and intensity (Cabeen *et al.* 2017). It is therefore possible that the methodology used in the current study was not sensitive enough to detect such subtle changes (Chapter 5). The methods in the current study focussed upon identifying a growth or survival phenotype at the population level in association with a particular stress. However, it might be productive to investigate the intensity and duration of a response to a specific stress by studying individual cells carrying a reporter of SigB activity. In addition, the current study utilised mutants lacking just one of the hypothetical stress sensing proteins, while the study by Cabeen *et al.* (2017) employed mutants lacking all but one of the RsbR proteins. Future work should combine both approaches in an attempt to identify the exact role of each protein in both stress sensing and in coordinating a response to the stress in conjunction with the other RsbR paralogues.

Prior to conducting investigations into the stress sensing capacities of the stressosome using deletion mutants, the effect of eliminating individual or combinations of proteins on the stoichiometry of the stressosome should first be assessed. The immunoprecipitation of the stressosome followed by liquid chromatography-tandem mass-spectrometry analysis described by Impens *et al.* (2017) was useful in predicting the likely components of the

stressosome, as RsbS, RsbT, Rsb and the RsbR paralogues all precipitated together. Therefore, repeating this technique using the deletion mutants in the presence of varying growth conditions and stresses may be useful in indicating whether the deletion of proteins from the stressosome leads to any secondary changes in stressosome composition. In addition, cryogenic electron microscopy and x-ray crystallography of both the wild-type and deletion mutants may be helpful in determining the quaternary structure of the stressosome. Previous work in *B. subtilis* has availed of cryogenic electron microscopy to determine the structure of the stressosome (Marles-Wright *et al.* 2008), however only electron micrographs have been presented for the *L. monocytogenes* stressosome (Impens *et al.* 2017).

It has been hypothesised that the stressosome is tethered to the cell membrane by a miniprotein, Prli42 (Impens *et al.* 2017). This hypothesis was developed through cellular fractionation, with Prli42 being found solely in the cell membrane fraction. Through the fluorescent tagging of the stressosome proteins, the cellular localisation of the complex may be determined. As noted by Stadler *et al.* (2013), the addition of a fluorescent tag to a protein can alter the cellular localisation of the protein, and therefore the protein should be tagged in at least two locations to minimise this effect. In addition, immunofluorescence can be used to confirm the results of fluorescent tagging (Stadler *et al.* 2013). The recently described cyclic-immunofluorescence enables the building of a 3D image of the cell, similar to that of cryogenic electron microscopy, with a the number of proteins that can be incorporated into the analysis theoretically unlimited (Lin *et al.* 2015, Galli *et al.* 2019). Determining the cellular localisation of the stressosome complex may be useful in determining the mechanism by which stress is sensed as it would enable certain theories to be deemed more likely than others.

Overall, there are still many outstanding questions regarding the role of RsbR and paralogues in stress sensing. Aside from Lmo0799, which is known to sense and respond to blue light photons (Ondrusch and Kreft 2011, Chan *et al.* 2013), the stimuli for each of the other stress sensors is unknown. For example, it is unclear whether the stressosome senses the increase in protons, the depletion of ATP associated with a decrease in pH or one of the many other pH-associated signals, if it indeed senses acid stress at all.

7.6 Conclusions

This project has provided insights into the role of SigB and the stressosome complex in the sensing and response of *L. monocytogenes* to environmental stresses. It has built upon previous investigations into the role of Lmo0799 as a blue light sensing protein, with a particular insight into the conserved cysteine residue at position 56 thought to be critical for blue light sensing. Investigations into the relationship between temperature and light sensing have uncovered possible evolutionary traits that prevent *L. monocytogenes* from sensing blue light within the host. It is possible that *L. monocytogenes* uses the presence of light, along with changes in temperature, to sense when it has entered or left the host. Transcriptomic investigations into whole genome transcriptomic changes in response to low intensity blue light exposure have provided evidence for the hypothesis that *L. monocytogenes* is able to sense and respond to secondary stresses associated with blue light exposure. Investigations into the role of RsbR and its paralogues in environmental stress sensing and virulence have identified likely redundancies between the proteins in stress sensing. Finally, the identification of conditions and compounds that influence the sensitivity of *L. monocytogenes* to killing by blue light provide important insights that will inform the development of blue light as a means on controlling this food-borne pathogen.

Chapter 8: Bibliography

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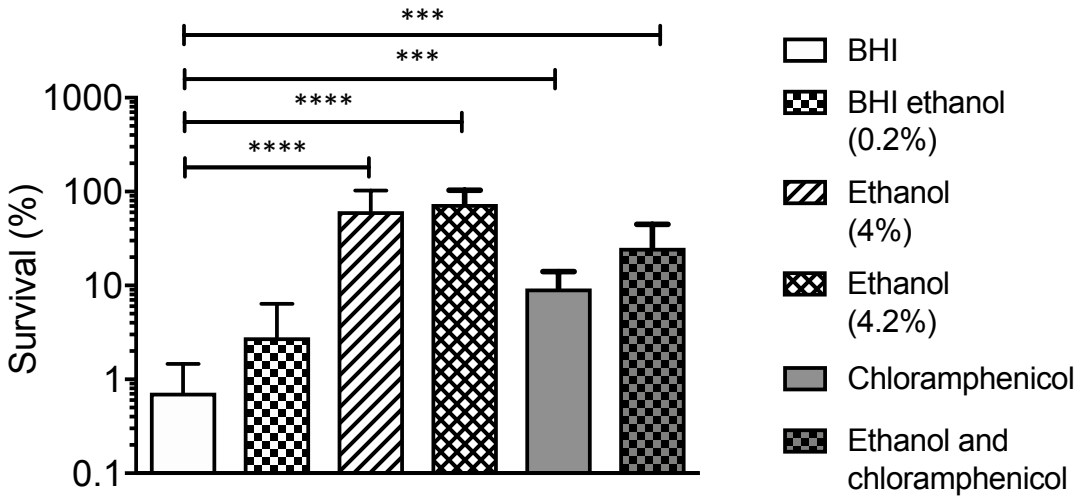
Appendix 1



Appendix 1. Sensitivity of *L. monocytogenes* to blue light is affected by pre-exposure to 4% ethanol, via a mechanism other than *de novo* protein synthesis.

Cultures were grown to early exponential phase at 37°C in BHI broth. Cells were centrifuged and resuspended in either BHI broth with and without chloramphenicol or BHI broth supplemented with 4% ethanol with or without chloramphenicol. Cells were incubated at 37°C for 1 h. Cells were centrifuged, washed once in PBS and resuspended in PBS, then exposed to 35 mW cm^{-2} 470 nm light for 4 h. Error bars represent SD from two independent replicates. Statistical significance was determined using a Kruskall-Wallis test with Dunn's multiple comparisons test (***: $p = \leq 0.001$; ****: $p = \leq 0.0001$).

Appendix 2

Overall, three peer-reviewed research articles, one book chapter, one conference review article, one oral presentation and three poster presentations were produced during this study.

Dorey, A. L., Giovannercole, F., Guerreiro, D., & Marinho, C. (2019). Microbial stress meeting: From systems to molecules and back. *New Biotechnology*, *49*, 66–70. https://doi.org/10.1016/J.NBT.2018.09.002

Dorey, A., Marinho, C., Piveteau, P., & O'Byrne, C. (2019). Role and regulation of the stress activated sigma factor Sigma B (σB) in the saprophytic and host-associated life stages of the pathogen *Listeria monocytogenes*. In *Advances in Applied Microbiology* (First Edit, pp. 1–48). Academic Press.

Dorey, A. and O'Byrne, C. P. Blue light sensing in *Listeria monocytogenes* is temperature dependent and the transcriptional responses is predominantly SigB-dependent. (In preparation).

Dorey, A., Crespo Tapia, N., den Besten, H., O'Byrne, C. P. and Abee, T. The role of alternative carbon sources in activating SigB and potential implications for food safety. (In preparation).

Dorey, A., Maher, F., Walsh, C. and O'Byrne, C. Ethanol exposure induces high levels of resistance to photodynamic inactivation in *Listeria monocytogenes.* (In preparation).

Dorey, A. L. (2018). 'The Impact of Temperature on the Response of *L. monocytogenes* to Visible Light'. *Foodborne Pathogens: From farm to pharmacy!* Kinsale, Ireland.

Dorey, A. L. and O'Byrne, C. P. (2018). 'The Sensing of Visible Light by *Listeria monocytogenes* in Temperature Dependent'. *Microbial Stress: from systems to molecules and back.* Kinsale, Ireland.

Dorey, A. L., Oliveira, A., NicAogáin, K., O'Donoghue, B., O'Byrne, C. P. (2017). 'The Effects of Growth Conditions and Secondary Environmental Stresses on the Response of *Listeria monocytogenes* to Visible Light'. *FEMS Congress.* Valencia, Spain.

Dorey, A. L., NicAogáin, K., O'Donoghue, B., Bennett, C., Conneely, A., O'Byrne, C. P. (2016). 'Investigation into the Response of *Listeria monocytogenes* to 470 nm Blue Light'. *International Symposium On Problems Of Listeria*. Paris, France.