Role and regulation of the stress activated sigma factor Sigma B (σ^B) in the saprophytic and host-associated life stages of *Listeria monocytogenes*.

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1. Abstract

The stress activated sigma factor sigma B (σ^B) plays a pivotal role in allowing the food-borne bacterial pathogen *Listeria monocytogenes* to modulate its transcriptional landscape in order to survive in a variety of harsh environments both outside and within the host. While we have a comparatively good understanding of the systems under the control of this sigma factor much less is known about how the activity of σ^B is controlled. In this review we present a current model describing how this sigma factor is thought to be controlled including an overview of what is known about stress sensing and the early signal transduction events that trigger its activation. We discuss the known regulatory overlaps between σ^B and other protein and RNA regulators in the cell. Finally, we describe the role of σ^B in surviving both saprophytic and host associated-stresses. The complexity of the regulation of this sigma factor reflects the significant role that it plays in the persistence of this important pathogen in the natural environment, the food-chain as well as within the host during the early stages of an infection. Understanding its regulation will be a critical step in helping to develop rational strategies to prevent its growth and survival in the food destined for human consumption and in the prevention of listeriosis.

2. Introduction

Listeria monocytogenes is a remarkable bacterial pathogen not only because of the sophisticated molecular mechanisms that it uses to invade and colonise the mammalian host (Cossart, 2011; de las Heras, Cain, Bielecka & Vazquez-Boland, 2011; Radoshevich & Cossart, 2018), but also because it is exquisitely well-adapted to cope with a range of environmental challenges including osmotic and acid stresses as well as cold temperatures (Gandhi & Chikindas, 2007; NicAogain & O'Byrne, 2016; O'Byrne & Karatzas, 2008; van Schaik & Abee, 2005). The latter properties make this food-borne pathogen particularly difficult to eliminate from the food chain, especially in so-called ready-to-eat foods, those foods that can be eaten without prior cooking (NicAogain & O'Byrne, 2016). Although infections are not very common in healthy individuals, the high mortality rate associated with infections (de Noordhout et al., 2014; Lecuit, 2007) combined with the ubiquity of this organism in the environment mean that is taken very seriously by food producers and it continues to represent a serious public health risk. A key step in developing improved food safety measures is to develop a mechanistic understanding of how this organism protects itself in the complex and challenging environments it encounters, both within the food chain and within the host. Such an understanding could then be used to inform the rational design of new control measures that target the Achilles heel of this pathogen, in order to prevent its survival and growth at critical points along the food chain.

A key step in adapting to new stresses in the environment is the reprogramming of the transcriptional landscape to align gene expression with the physiological needs of the cell, and this is achieved by a panoply of both protein and ribonucleic acid transcriptional regulators. At the top of the hierarchy of transcriptional regulation lie the sigma factors, which largely determine the genes that are transcribed at any time by directing the transcriptional machinery to the appropriate promoter sequences. Most *L. monocytogenes* strains have 5 sigma factors, including the principal housekeeping sigma factor σ^A and four alternative sigma factors, σ^B, σ^C, σ^H, and σ^L (Glaser et al., 2001; O'Byrne & Karatzas, 2008). σ^B is the factor that controls the general stress response in *L. monocytogenes* and of the four alternative

sigma factors it has the largest regulon, with almost three hundred genes (approximately 10% of the genome) under the positive control of this sigma factor (Chaturongakul et al., 2011). Wiedmann and colleagues identified the *sigB* locus based on homology with the σ^B in *B. subtilis* and demonstrated an important role for this sigma factor in acid tolerance (Wiedmann, Arvik, Hurley & Boor, 1998). Almost simultaneously Becker et al., (1998) identified the same locus and showed the involvement of σ^B in the response to osmotic stress.

Subsequently, σ^B in *L. monocytogenes* has received a lot of research attention with several studies helping to define fully the regulon (Abram et al., 2008a; Abram et al., 2008b; Kazmierczak, Mithoe, Boor & Wiedmann, 2003; Raengpradub, Wiedmann & Boor, 2008; Toledo-Arana et al., 2009; Wemekamp-Kamphuis et al., 2004; Wurtzel et al., 2012). Genes under σ^B control are known to contribute to a variety of stress resistance mechanisms including osmoregulation (Cetin, Zhang, Hutkins & Benson, 2004; Fraser, Sue, Wiedmann, Boor & O'Byrne, 2003; Sue, Boor & Wiedmann, 2003) acid tolerance (Cotter, Gahan & Hill, 2001; Wemekamp-Kamphuis et al., 2004; Wiedmann, Arvik, Hurley & Boor, 1998), bile tolerance (Begley, Sleator, Gahan & Hill, 2005; Zhang et al., 2011), cell wall acting antimicrobials (Begley, Hill & Ross, 2006), and visible light (O'Donoghue et al., 2016; Ondrusch & Kreft, 2011; Tiensuu, Andersson, Rydén & Johansson, 2013). Its role in surviving the gastrointestinal phase of the infectious cycle is also now well established (Dowd, Joyce, Hill & Gahan, 2011; Garner, Njaa, Wiedmann & Boor, 2006; Sleator, Watson, Hill & Gahan, 2009; Toledo-Arana et al., 2009). There is also substantial evidence that σ^B plays an important role in virulence. First, the gene encoding the virulence master regulator PrfA is preceded by two overlapping promoters, one of which is recognised by σ^B (Kazmierczak, Wiedmann & Boor, 2006; Rauch, Luo, Muller-Altrock & Goebel, 2005). Second, the *inlAB* operon that encodes the cell invasion proteins internalin A and B is under σ^B control (Kim, Marquis & Boor, 2005; Kim, Gaidenko & Price, 2004). Overall the current view is that σ^B plays a vital role in the early gastrointestinal stages of the infection whereas the regulator PrfA dominates during systemic spread and the intracellular stages of the infectious cycle (de las Heras, Cain, Bielecka & Vazquez-Boland, 2011; O'Byrne & Karatzas, 2008).

While there is certainly still lots to learn about the systems under σ^B control and the roles they play in survival of this pathogen in the environment, the biggest outstanding questions relate to the mechanisms that control the activation of σ^B. Understanding the details of how σ^B becomes activated will be a critical step in developing strategies to undermine its protective functions and ultimately to prevent this pathogen from surviving in the human and animal food chains. This review attempts to review our current understanding of the σ^B system in *L. monocytogenes* including the sensory mechanisms that trigger its activation. We also discuss the regulatory overlap between this sigma factor and other important global regulators, including small regulatory RNAs, in this pathogen. The role that it plays in both the saprophytic and virulence phases of the life cycle of *L. monocytogenes* are also presented, along with some of the outstanding questions and challenges in this field.

3. The current model of σ^B 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 σ^B signalling cascade (Martinez, Reeves & Haldenwang, 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 σ^B signalling cascade (Ferreira, Gray, Wiedmann & Boor, 2004). Therefore, research into the stressosome complex and σ^B signalling cascade in *B. subtilis* has provided a solid foundation to guide research into the same areas within *L. monocytogenes.*

3.1 The σ^B signalling cascade

In the unstressed *B. subtiis* cell, σ^B is sequestered by the anti-sigma factor RsbW (**R**egulator of **S**igma **B**), inhibiting its interaction with RNA polymerase, and the σ^B regulon is not transcribed (Ferreira,

O'Byrne & Boor, 2001). Upstream of RsbW, the anti-anti-sigma factor, RsbV, is phosphorylated and unable to bind RsbW in its phosphorylated state (Yang, Kang, Brody & Price, 1996). In a stressed cell, RsbU acts as a phosphatase, dephosphorylating RsbV, and enabling the binding of RsbV to RsbW (Yang, Kang, Brody & Price, 1996). As a result, σ^B is left free to bind to the core enzyme of RNA polymerase, and transcription of the σ^B regulon occurs (Hecker, Pané-Farré & Völker, 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 σ^B signalling cascade in response to environmental stress (Fig. 1B) (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, Lewis, Harris, Yudkin & Delumeau, 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.

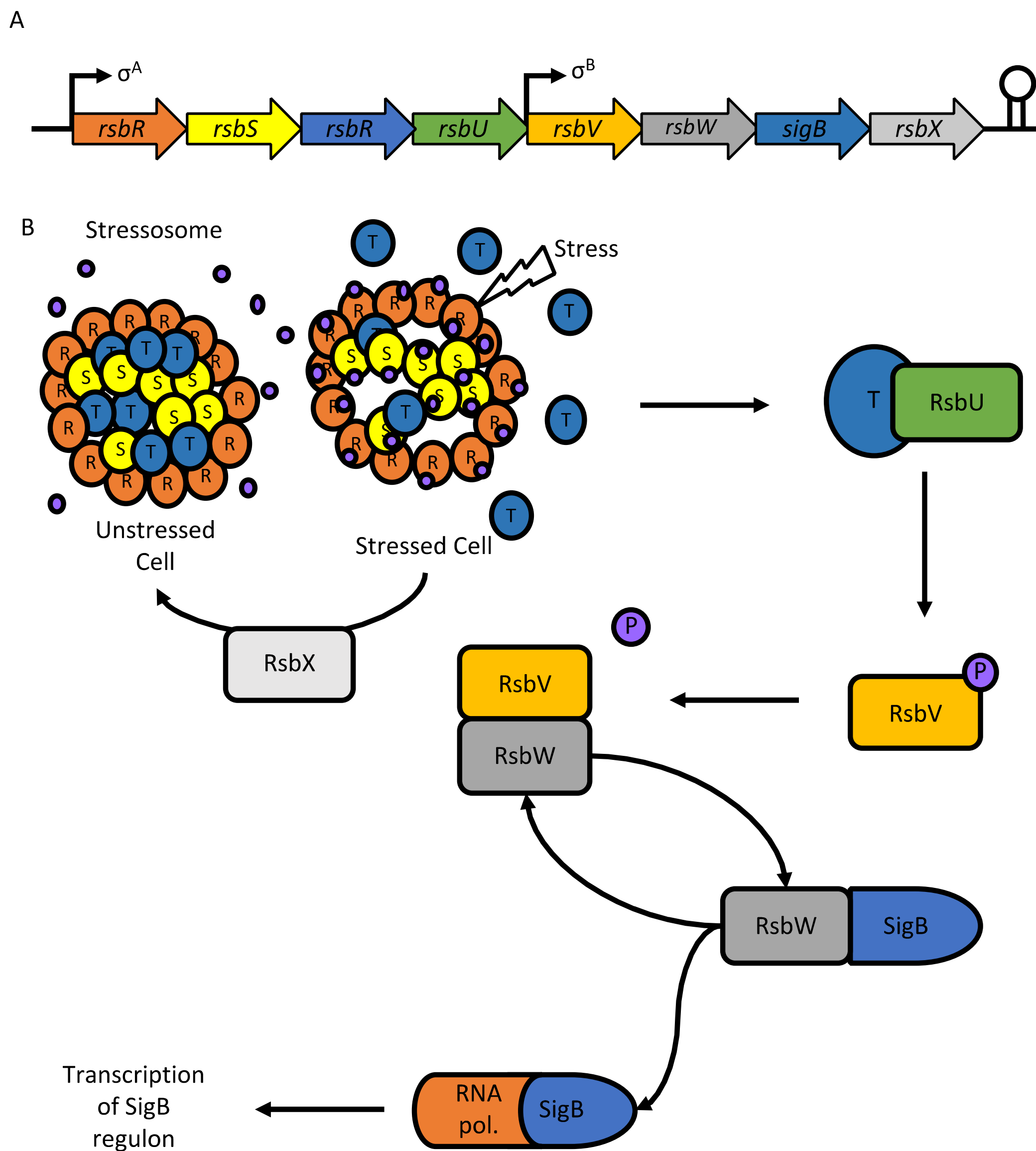

Fig. 1 Overview of the *sigB* operon and model of the σ^B regulatory mechanism. (A) The *sigB* operon of *L. monocytogenes*. Each gene in the *sigB* operon is represented by an open arrowhead. Transcription can be initiated from either of the two promoters represented by angled arrowheads, and the putative terminator sequence is represented by a stem and loop structure. (B) Upon perception of a stress signal RsbR and RsbS are phosphorylated through the kinase activity of RsbT, and this causes RsbT dissociate from the stressosome complex, making it available to bind to RsbU. RsbU then becomes active as a phosphatase thereby facilitating the dephosphorylation of the anti-anti sigma factor RsbV. The anti-sigma factor, RsbW, is bound to σ^B in an unstressed cell, but has a higher affinity

for RsbV in its unphosphorylated state, leading to its dissociation from σ^B in a stressed cell. Sigma B is then free to bind to RNA polymerase, and initiate transcription of the σ^B gene regulon.

The continual activation of σ^B is deleterious to the cell, and mutants with constitutively phosphorylated RsbS generate small colonies (Min Kang, Brody, Akbar, Yang & Price, 1996). 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, Lewis, Harris, Yudkin & Delumeau, 2003). Computational modelling of RsbX phosphatase activity suggests that RsbX dephosphorylates RsbS at a higher rate than it does RsbR (Liebal, Millat, Marles-Wright, Lewis & Wolkenhauer, 2013).

3.1.1 RsbU

RsbU is the first protein in the σ^B 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, 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 energy stress through σ^B activation (Shin, Brody & Price, 2010). While these results show an essential role for RsbU in responding to energy stress via activation of σ^B, 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 & Boor, 2004).

3.1.2 RsbV

Downstream from RsbU, in the unstressed cell, the anti-anti-sigma factor RsbV exists in its unphosphorylated 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 & Boor, 2004), suggesting that RsbV is required for the activation of σ^B 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 σ^B activity via RT-PCR targeted against the σ^B-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 & Boor, 2006). Taken together, these studies show a requirement for RsbV to activate σ^B in response to certain environmental stresses. In the absence of RsbV, it is likely that RsbW remains bound to σ^B, and therefore σ^B 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 & 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 & 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 σ^B (O'Byrne & Karatzas, 2008). In the presence of σ^B, 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.

3.2 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 & Lewis, 2010; Pane-Farre, Quin, Lewis & Marles-Wright, 2017). 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 & Lewis, 2010; Pane-Farre, Quin, Lewis & Marles-Wright, 2017). 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. 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 (Heavin & O'Byrne, 2012; Ondrusch & Kreft, 2011).

4. 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 & Kreft, 2011). Novel work using N-terminomics has identified a previously undescribed miniprotein, Prli42, which is essential for σ^B 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, Delumeau & Lewis, 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, Gaidenko & Price, 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, Gaidenko & Price, 2004). More recent work has shown that cells expressing only a single paralogue of RsbR have an altered response profile to ethanol stress (Cabeen, Russell, Paulsson & Losick, 2017). While all mutants were able to activate σ^B 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, Russell, Paulsson & Losick, 2017).

4.1 Lmo0799 as a blue light sensing protein

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 & Kreft, 2011). Through sequence comparison, it has been noted that YtvA and Lmo0799 share 54% homology (Chan, Lewis & Bogomolni, 2013), and several amino acids that are required for the functioning of YtvA as a blue light sensor are conserved in Lmo0799 (Ondrusch & 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, Kim, Weigel, Brody & Price, 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, Polverini, Quest & Gärtner, 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 & 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 & 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 & 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, Polverini, Quest & Gärtner, 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 σ^B in response to blue light irradiation, and its alteration to either a serine or alanine residue inhibits σ^B activation in response to blue light irradiation (Ávila-Pérez, Hellingwerf & Kort, 2006; Gaidenko, Kim, Weigel, Brody & Price, 2006). Likewise, the conversion of the conserved cysteine residue in Lmo0799 (Cys56) (Ondrusch & 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 σ^B in *L. monocytogenes* in response to blue light irradiation.

While the induction of the σ^B-dependent genes *lmo0596* and *lmo2230* in response to light requires Lmo0799 (Tiensuu, Andersson, Rydén & Johansson, 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 are able to sense and respond to alternative stresses associated with

photodynamic inactivation (PDI) via an alternative sensory mechanism. In addition, while σ^B 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 a variable role for σ^B in blue light resistance, a phenotype that has previously been reported for resistance to oxidative stress (Boura et al., 2016). Despite evidence suggesting that growth inhibition of *L. monocytogenes* by visible 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 & Kreft, 2011).

5. Role of σ^B under different stresses

The role of σ^B in coordinating the response to osmotic stress in *L. monocytogenes* was first reported by Becker *et al.* (1998). Since this, σ^B has been implicated in the resistance of *L. monocytogenes* to a plethora of environmental stresses, including, but not limited to, osmotic (Fraser, Sue, Wiedmann, Boor & O'Byrne, 2003; Utratna, Shaw, Starr & O'Byrne, 2011), pH (Wemekamp-Kamphuis et al., 2004; Wiedmann, Arvik, Hurley & Boor, 1998), temperature (Liu, Graham, Bigelow, Morse & Wilkinson, 2002) and oxidative stress (Ferreira, O'Byrne & Boor, 2001). In addition to its role in initiating the general stress response, σ^B is also implicated in the formation of biofilms by *L. monocytogenes* (van der Veen & Abee, 2010).

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, Lawrence, Ainsworth & Austin, 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 (O'Byrne & Booth, 2002; Tombras Smith, 1996).

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, Smith & Smith, 1994). The primary glycine betaine uptake system is encoded by *betL* (Sleator, Gahan, Abee & Hill, 1999), which has a σ^B promoter -33 bases upstream (Fraser, Harvie, Coote & O'Byrne, 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, Gahan, Abee & Hill, 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 σ^B in glycine betaine accumulation (Fraser, Sue, Wiedmann, Boor & O'Byrne, 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 σ^B promoter upstream of *opuCA,* with carnitine uptake being completely abolished in the Δ*opuC* mutant (Fraser, Harvie, Coote & O'Byrne, 2000). Similarly to the Δ*opuC* mutant, carnitine uptake is almost completely abolished in the Δ*sigB* mutant (Fraser, Sue, Wiedmann, Boor & O'Byrne, 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, Shaw, Starr &

O'Byrne, 2011). Carnitine transport has also been shown to be important for growth and survival in the murine gastrointestinal tract (Sleator & Hill, 2010; Sleator, Wouters, Gahan, Abee & Hill, 2001).

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 less acidic 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, Gahan & Hill, 2001), arginine deaminase system (Ryan, Begley, Gahan & Hill, 2009), and the adaptive acid tolerance response (ATR) (Davis, Coote & O'Byrne, 1996; O'Driscoll, Gahan & Hill, 1996), all of which, at least partially, require σ^B.

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 (Gahan & Hill, 2014), a decarboxylation reaction that consumes a proton and so contributes to reducing the acidity of the cell cytoplasm (Karatzas, Brennan, Heavin, Morrissey & O'Byrne, 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, Gahan & Hill, 2001). When transcription of the *gad* genes was measured in the Δ*sigB* mutant, only transcription of *gadE* was induced under the same conditions, confirming the functionality of the σ^B 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, Sue, O'Byrne & Boor, 2003). When considered together, these results suggest that while σ^B is involved in regulating the GAD system, there is likely to 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, Begley, Gahan & Hill, 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 & Hill, 2014). The conversion of arginine to citrulline is regulated by σ^B via the ArcA protein; ArcA is encoded by *lmo0043* which has a σ^B 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, Coote & O'Byrne, 1996). In the absence of both σ^B 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, O'Byrne & Boor, 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, O'Byrne & Boor, 2001). In a similar study, the role of σ^B in the ATR was assessed at different phases of growth, with a greater requirement for σ^B identified as the cells approached stationary phase compared to exponential phase (Ferreira, Sue, O'Byrne & Boor, 2003).

From the studies presented, it is clear that σ^B 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 mechanisms utilised by *L. monocytogenes* upon encountering acid stress require a number of complex pathways, likely involving number of regulators in addition to σ^B. More research will be necessary to fully elucidate the regulatory interactions that occur during acid stress and to clarify fully the role of σ^B in the response.

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, Sahiner, Sackesen, Erzurum & Kalayci, 2012). The three most physiologically important categories of ROS include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) (Imlay, 2013). The negative consequences of ROS on cells include DNA damage, lipid peroxidation, and oxidative damage of proteins (Bandyopadhyay, Das & Banerjee, 1999); for a recent review see (Imlay, 2018).

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, Sahiner, Sackesen, Erzurum & Kalayci, 2012). Research investigating the role of σ^B 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 σ^B is at least semi-responsible for resistance to oxidative stress (Ferreira, O'Byrne & Boor, 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 σ^B 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 σ^B by oxidative stress. Despite this evidence for the role of σ^B in oxidative stress tolerance, Boura et al. (2016) have shown that the presence of σ^B is deleterious for the cell at stationary phase in the presence of hydrogen peroxide. This apparent paradox shows that further research will be required to clarify to involvement of σ^B in the response to oxidative stress.

5.4 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, Teixeira, Rosa, Oliveira & Azeredo, 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 & Martin, 2005). Two papers confirm a role for σ^B in biofilm formation (Lemon, Freitag & Kolter, 2010; van der Veen & Abee, 2010). The study by van der Veen and Abee (2010) identified significant levels of σ^B 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 & Abee, 2010). In agreement with this, Lemon et al. (2010) confirmed a requirement for σ^B in biofilm formation at 30°C only (Lemon, Freitag & Kolter, 2010). The results of these studies are indicative of a variable, and possibly temperature-dependent role for σ^B 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 (Toledo-Arana et al., 2009) in microtitre plates (Lemon, Higgins & Kolter, 2007). σ^B 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), another study 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 (Todhanakasem & Young, 2008). However, in agreement with Lemon et al. (2007) , this study identified a requirement for flagella in biofilm formation in microtitre plates (Todhanakasem & Young, 2008). When the results of these studies are considered together, they indicate a role for σ^B in biofilm formation that depends on the environmental conditions encountered.

It has been known for over 30 years that *L. monocytogenes* only expresses flagella at temperatures below 37°C (Peel, Donachie & Shaw, 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 (Grundling, Burrack, Bouwer & Higgins, 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 & Higgins, 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 & Higgins, 2011).

A previous study has shown that the Δ*sigB* mutant is motile at 37°C, contrasting with the wild-type which loses motility at 37°C (Raengpradub, Wiedmann & Boor, 2008). In the absence of σ^B motility is increased, despite a σ^B promoter upstream of several flagellar biosynthesis genes (Toledo-Arana et al.,

2009), 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 likely, that σ^B is required for a role other than regulation of motility in biofilm formation.

6. Role of σ^B in virulence

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 σ^B-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 σ^B promoter and a PrfA binding site were altered in a PrfA-dependent but σ^B-independent manner (Toledo-Arana et al., 2009). This alteration in transcriptional regulation indicates a complex overlap between PrfA and σ^B in virulence gene expression and suggests a switch between these regulators as the infection progresses beyond the gastrointestinal tract.

6.1 A role of σ^B in colonizing the host

The first step in colonising the mammalian host is the successful transit through the extremely acidic conditions encountered in the stomach. As discussed in section 5.2 σ^B plays a significant role in acid tolerance through its involvement in regulating expression of the GAD and ADI systems. Mutants lacking a fully functional GAD system show reduced virulence in a mouse model. Specifically mutants lacking one or more of the glutamate decarboxylase genes were less capable of infecting the spleen and liver of mice that were challenged by direct gastric gavage (Feehily et al., 2014). Somewhat surprisingly however the number of *L. monocytogenes* cells present in the faeces of the infected

animals was not affected by loss of the GAD system. The ADI system was also found to contribute to virulence since mutants lacking *arcA*, which encodes arginine deiminase, colonised the spleen of mice less efficiently than the wild-type after intraperitoneal inoculation (Ryan, Begley, Gahan & Hill, 2009).

From the stomach, *L. monocytogenes* is transported to the gastrointestinal tract where the processes of adhesion and internalisation begin (Mengaud, Ohayon, Gounon, Mege R-M & Cossart, 1996; Vazquez-Boland et al., 2001). InlA and InlB are two of the internalin proteins produced by *L. monocytogenes,* under the control of σ^B (Kazmierczak, Mithoe, Boor & Wiedmann, 2003), enabling the cells to bind to human E-cadherin and Hepatocyte Growth Factor Receptor (HGFR) proteins (Bonazzi, Lecuit & Cossart, 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, Bierne & Cossart, 2006). Likewise, the binding of InlB to HGFR also induces cytoskeletal rearrangements but, in addition, facilitates clatherin-mediated endocytosis (Bierne, Sabet, Personnic & Cossart, 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, Marquis & Boor, 2005).

The OpuC operon of *L. monocytogenes* is involved in the uptake of carnitine and glycine betaine in response to osmotic stress (Sleator, Wouters, Gahan, Abee & Hill, 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, Wouters, Gahan, Abee & Hill, 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* colonisation of the host (Wemekamp-Kamphuis et al., 2004).

In the gastrointestinal tract, bile stress is one of the stresses experienced by the pathogen, with a role for σ^B in bile tolerance clearly defined (Gahan & Hill, 2014; Sue, Boor & Wiedmann, 2003; Zhang et al., 2011). Both *bsh*, encoding bile salt hydrolase, and *bilE*, a putative bile efflux system, have been shown to be under the transcriptional control of σ^B (Begley, Sleator, Gahan & Hill, 2005; Fraser, Sue, Wiedmann, Boor & O'Byrne, 2003; Sue, Boor & Wiedmann, 2003). Indeed mutants lacking σ^B are exquisitely sensitive to bile (Begley, Sleator, Gahan & Hill, 2005; Zhang et al., 2011). Interestingly, 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, Sleator, Casey, Hill & Gahan, 2009). The mechanism behind this observation hasn't yet been defined but one possibility is that bile might perturb osmoregulation and the compatible solute carnitine could help to mitigate this effect.

6.2 Overlap between σ^B and PrfA in virulence

During transition from the saprophytic to virulent state, *L. monocytogenes* relies upon complex regulatory networks that fine-tune the expression of virulence factors in response to environmental signals (Gray, Freitag & Boor, 2006). In this context, an interconnection between the pleiotropic σ^B and PrfA transcriptional regulators is important both for the transition from environment to host and in establishing an infection (Chaturongakul, Raengpradub, Wiedmann & Boor, 2008; O'Byrne & Karatzas, 2008; Ollinger, Bowen, Wiedmann, Boor & Bergholz, 2009).

The transcription of *prfA* can be initiated from three different promoter sites. The promoter P_{plcA} is located upstream of *plcA* and can initiate the synthesis of a bisistronic mRNA comprising *plcA* and *pfrA* (Camilli, Tilney & Portnoy, 1993). The other two alternative promoter sites $P1_{prfA}$ and $P2_{prfA}$ are located immediately upstream of *prfA* (Freitag, Rong & Portnoy, 1993). The first ($P1_{prfA}$) is recognized

only by the *L. monocytogenes* housekeeping sigma factor σ^A , while $P2_{prfA}$ consists of two overlapping promoters, one recognized by σ^A and the other by σ^B (Nadon, Bowen, Wiedmann & Boor, 2002). Moreover, the activity of the $P2_{prfA}$ promoter region was shown to contribute to the majority of the *prfA* transcripts in both intra- and extracellular bacteria (Kazmierczak, Wiedmann & Boor, 2006). In the presence of active PrfA, σ^B is responsible for reduced expression of the PrfA regulon. Therefore, the regulation of PrfA activity by σ^B is exerted either by transcriptional activation of the $P2_{prfA}$ promoter or by post-transcriptional downregulation of the PrfA regulon expression. Interactions between PrfA and σ^B ensure the rapid induction of regulon expression to facilitate infection and virulence, as well as subsequent downregulation to avoid overexpression of virulence genes, reducing cytotoxic effects (Ollinger, Bowen, Wiedmann, Boor & Bergholz, 2009). The regulatory basis for the interactions between these systems are discussed further in section 7.

7. Crosstalk between σ^B and other regulators in *Listeria monocytogenes*

While the σ^B regulon has been well studied and defined, the interaction and overlap of σ^B with other transcriptional regulators is less well understood. Here we explore some of the known interactions between σ^B and other protein and RNA regulators.

7.1. σ^B-dependent small regulatory RNAs

Small non-coding RNAs (sRNAs) are untranslated transcripts that base pair to target mRNAs at specific regions of complementarity, and control biological functions by regulating gene expression at the post-transcriptional level. In pathogenic bacteria, relatively short RNA transcripts (about 50 to 500 nucleotides) are established as important gene regulators involved in post-transcriptional control of cellular processes such as metabolism, stress response and virulence (Waters & Storz, 2009). Several studies aimed at defining the whole transcriptome of *Listeria* have been reported on its small RNome

(Mraheil et al., 2011; Oliver et al., 2009; Toledo-Arana et al., 2009; Wurtzel et al., 2012). Overall, the genome of *L. monocytogenes* EGD-e includes 304 sRNAs, of which 154 are proposed to be *trans*-acting sRNAs, 46 *cis*-acting regulatory elements and 104 anti-sense RNA (asRNAs) (Becavin et al., 2017).

The transcription of several small regulatory RNAs of *L. monocytogenes* depends on σ^B (Table 1).and they each possess a σ^B-dependent promoter upstream of their coding region. This group includes sRNAs of all classes: three *trans*-acting sRNAs (SbrA, Rli47 and Rli33-1), two anti-sense RNAs (Anti-LhrC-5 and Anti2270) and a *cis*-acting regulatory element (Rli95) (Mraheil et al., 2011). Additionally, σ^B can also control sRNAs via the σ^B-dependent RNA-binding protein Hfq, which itself modulates the stability or translation of mRNAs while contributing to stress tolerance and virulence in *L. monocytogenes* (Christiansen, Larsen, Ingmer, Sogaard-Andersen & Kallipolitis, 2004). Here, we highlight a selection of σ^B-dependent sRNAs that contribute to *L. monocytogenes* stress tolerance and adaptation to saprophytic and virulence-specific niches.

7.1.1. Trans-acting sRNAs directly dependent on σ^B

Trans-acting RNAs are generally small non-coding RNAs, located at a distance from the genes encoding their target mRNAs. Many of these sRNA-mRNA interactions occur near the ribosome-binding site (RBS) of the target mRNAs. This can lead to translation inhibition through occlusion of the Shine-Dalgarno (SD) site, and/or to transcript degradation by targeting the RNA duplex structure for degradation by ribonucleases (RNases). Alternatively, some sRNAs can base pair to a more distant location and increase the ribosome binding by preventing the formation of a secondary inhibitory structure (Storz, Vogel & Wassarman, 2011).

SbrA: SbrA (also known as Rli11) is a 70-nucleotide long sRNA, encoded in the intergenic region between *lmo1374* and *lmo1375*. Using bioinformatic tools, Nielsen and collaborators (2008) identified

a σ^B-dependent promoter upstream each of the four Sigma B-dependent RNAs *sbrA-D* in *L. monocytogenes* EGD-e genome (Nielsen et al., 2010). Of the four putative sRNAs identified as potentially σ^B-dependent, σ^B-dependent expression was confirmed *in vivo* only in SbrA after experimental validation. SbrA was found highly conserved in sequenced *Listeria* species and highly expressed in stationary phase *L. monocytogenes* EGD-e cells cultured in rich medium (Nielsen et al., 2010; Toledo-Arana et al., 2009). However, RNA-seq analysis revealed that the transcript levels for this sRNA were not σ^B-dependent in *L. monocytogenes* 10403S (Oliver et al., 2009). Since no hypothetical target mRNA was successfully identified, and no function on growth or survival under harsh conditions such as low temperature, osmotic, acid and alcohol stress was identified, the role of SbrA remains to be determined (Nielsen, Olsen, Bonde, Valentin-Hansen & Kallipolitis, 2008).

Rli47: Rli47 (also referred to as SbrE) is a ~500 nucleotides long sRNA, located in the intergenic region between *lmo2141* and *lmo2142* of *L. monocytogenes* (Toledo-Arana et al., 2009). Conserved among the 18 *L. monocytogenes* genomes, Rli47 was assigned to the σ^B regulon after a σ^B-dependent promoter was identified upstream of the coding sequence. Several studies reported Rli47 as being highly transcribed in stationary phase *L. monocytogene*s cells using RNA-seq (Oliver et al., 2009), tiling microarray study (Toledo-Arana et al., 2009), and quantitative RT-PCR (Mujahid, Bergholz, Oliver, Boor & Wiedmann, 2012). Rli47 was found to be highly expressed in stationary phase cells exposed to oxidative stress, although this was not consistent with phenotypic data (Mujahid, Bergholz, Oliver, Boor & Wiedmann, 2012). The fact that Rli47 is induced in the intestinal lumen (Toledo-Arana et al., 2009) and macrophages (Mraheil et al., 2011), highlights a possible involvement of this sRNA in virulence processes. Moreover, a role in adaptation to environmental conditions has been suggested, perhaps involving crosstalk between σ^B and the AgrA regulons, since a higher level of Rli47 transcripts was reported in a *L. monocytogenes* EGD-e Δ*agrA* mutant strain during soil survival (Vivant, Garmyn, Gal, Hartmann & Piveteau, 2015). Microarray and proteomics experiments identified lower transcript levels of the gene *lmo0636* and reduced levels of the proteins Lmo0637 (methyltransferase) and

Lmo2094 (L-fuculose-phosphate aldolase) in the *rli47* isogenic mutant (Mujahid, Bergholz, Oliver, Boor & Wiedmann, 2012). However, no phenotype in growth or survival under a variety of environmental stress conditions has yet been associated with Rli47 and, despite all efforts, the specific function of this σ^B-dependent sRNA remains unknown.

Rli33-1: The LhrC is the largest multicopy family of sRNAs in *L. monocytogenes.* It consists of seven sibling sRNAs, five highly homologous sRNAs LhrC1-5 as well as Rli22 and Rli33-1, which are both structurally and functionally related to the LhrCs but have lower homology. With regulatory roles under virulence conditions, all seven sibling sRNAs are expressed from individual promoters, of which the *lhrC1*-5 and *rli22* are positively regulated by the two-component system LisRK, while the expression of *rli33*-1 is under σ^B control (Mollerup et al., 2016). In contrast to the other six LhrC sibling sRNAs, the expression of Rli33-1 is not induced by heme toxicity (Dos Santos et al., 2018). Alongside LhrC1-5, the expression of Rli33-1 is required for *L. monocytogenes* infection of macrophages (Mraheil et al., 2011). Moreover, it is also involved in the post-transcriptional repression of three targets, *oppA*, encoding a virulence-associated oligo-peptide binding protein, *lapB,* encoding a cell wall anchored virulence adhesion, and *tcsA,* encoding a $CD4^+$ T cell-stimulating antigen, via base pairing (Mollerup et al., 2016). Together, these findings suggest a regulatory function for Rli33-1 in the intracellular environment.

7.1.2. Anti-sense sRNAs Anti-LhrC-5 and Anti2270

Anti-sense small RNAs are encoded opposite to annotated open reading frames (ORFs), which enclose considerable base complementarity. Generally, the sRNA-mediated regulation inhibits mRNA transcription and/or translation or induces their rapid degradation, although it can also activate the expression of target mRNAs in some specific cases. Particularly in *Listeria*, asRNAs are known to regulate a variety of functions such as virulence, toxins, motility, and biofilm formation (Caldelari,

Chao, Romby & Vogel, 2013; Wurtzel et al., 2012). Several long and short asRNAs were previously identified in *Listeria* (Toledo-Arana et al., 2009; Wurtzel et al., 2012). Although asRNAs can regulate gene expression via effects on gene transcription, mRNA stability or translation (Brantl, 2007), not much is known about the function of the σ^B–dependent anti-sense sRNAs in *L. monocytogenes*. Besides being over-expressed in hypoxia (Toledo-Arana et al., 2009), the Anti-LhrC-5 (Anti0946) is a LhrC-5 homologue that has a σ^B promoter and whose coding sequence overlaps the sequences of the sRNA LhrC-5 and *lmo0946* (Mraheil et al., 2011). Similarly, Anti2270 is an antisense sRNA partially encoded in the intergenic region between *lmo2269* and *lmo2270* (competence transcription factor ComK', N terminal), overlapping the 5'-UTR of the last. Moreover, a putative σ^B promoter was identified upstream from this sequence as well (Mraheil et al., 2011), but its function remains to be discovered.

7.1.3. Cis-encoded sRNA Rli95

The *cis*-encoded sRNA Rli95 is the only σ^B-dependent sRNA of its class. It has been proposed to be involved in virulence, as it is known to be upregulated in macrophages (Mraheil et al., 2011). Rli95 was recently identified as one of the two *Listeria* guanine riboswitches (Krajewski, Isoz & Johansson, 2017). Its transcriptional termination is induced by the purine analogue 6-N-hydroxylaminopurine, hence preventing the expression of its downstream genes *lmo1885* and *lmo1884* that encode a xanthine phosphoribosyl transferase and a xanthine permease, respectively.

7.2. Indirect σ^B regulation of sRNAs expression via RNA chaperone Hfq

The interaction between a *trans*-acting sRNA and its targets often relies on a RNA chaperone such as Hfq, which promotes sRNA-mRNA duplex formation and stability. Thus, the riboregulation function of several sRNAs relies on the activity of Hfq. Although its role is well established in Gram-negative bacteria, its function has been less studied in Gram-positive bacteria and diverges between species

(Bouloc & Repoila, 2016). In *L. monocytogenes*, a σ^B-regulated promoter located in the *hfq* upstream region explains its σ^B-dependent expression. In fact, Hfq was shown to contribute to survival in harsh conditions, such as osmotic and ethanol stress, stationary growth phase, as well as long-term survival under amino acid-limiting conditions. Furthermore, Hfq seems to play a role in virulence by contributing to *L. monocytogenes* pathogenicity in mice, but surprisingly not in the infection of cultured cell lines (Christiansen, Larsen, Ingmer, Sogaard-Andersen & Kallipolitis, 2004). Hfq-binding sRNAs were discovered using co-immunoprecipitations followed by enzymatic RNA sequencing. This approach allowed the identification of three Hfq-binding regulatory small RNA in *L. monocytogenes*, LhrA, B and C (Christiansen et al., 2006). Specifically, Hfq not only stimulates and stabilizes the base pairing of LhrA to its target Shine-Dalgarno sequence, but also controls the translation and degradation of the target mRNAs *chiA*, which encodes a chitinase, and *lmo0302*, which encodes a hypothetical protein (Nielsen et al., 2011). It was recently shown that the number of arginines in a semi-conserved patch on the rim of a Hfq hexamer increases its RNA annealing activity, although Gram-positive Hfq proteins showed little or no activity at all (Zheng, Panja & Woodson, 2016).

7.3. The σ^B regulatory network overlaps with multiple other regulons

Crosstalk between regulatory networks is important in bacteria as it allows improved specificity of signal detection and helps to fine-tune the amplitude of the transduced signal to the precise environmental conditions encountered. Regulatory networks are likely to be crucial for the appropriate expression of stress response and virulence genes in *L. monocytogenes*. Over the years, transcriptomic and phenotypic data strongly suggest a crucial role for σ^B in modulating the transcriptional networks of *L. monocytogenes* during both the saprophytic and host-associated stages of its life cycle. Several studies point towards the largest regulon overlaps occurring between σ^B and other transcriptional regulators such as AgrA, CodY, CtsR, HrcA, MogR, PrfA and other sigma factors (Chaturongakul et al., 2011; Garmyn, Augagneur, Gal, Vivant & Piveteau, 2012; Guariglia-Oropeza et

al., 2014; Hu et al., 2007; Lobel & Herskovits, 2016). Whole genome microarray analyses in *L. monocytogenes* 10403S showed considerable overlap among σ^B and others regulons (Chaturongakul et al., 2011). Each overlapping regulon also included genes categorized into multiple biological function categories, highlighting the complexity of this regulatory network. Overall, complex regulatory networks may allow *L. monocytogenes* to rapidly fine-tune its gene expression in response to the ever-changing environments, and integrate various stimuli on the regulation of specific phenotypic responses.

7.3.1. Agr

First described in *S. aureus* (Recsei et al., 1986), the accessory gene regulator Agr is an auto-inducible transcriptional regulator associated with virulence and biofilm formation (Lauderdale, Boles, Cheung & Horswill, 2009). The *agr* locus comprises two different transcriptional units, RNAII and RNAIII, which are transcribed from the $P2_{agr}$ and $P3_{agr}$ promoters, respectively (Thoendel, Kavanaugh, Flack & Horswill, 2011). Activation of *agr* promoters is the primary function of RNAII gene products, which are significantly aided by the staphylococcal accessory gene regulator SarA (Cheung, Bayer, Zhang, Gresham & Xiong, 2004). While RNAII encodes the four-gene operon *agrBDCA*, the RNAIII is the effector molecule of the *agr* system, with a dual role in *S. aureus*. It has been suggested that the RNAIII acts in *trans*, regulating its targets at the post-transcriptional level by binding antisense to target mRNAs and occluding the Shine-Dalgarno sequence (Boisset et al., 2007). Additionally, it also contains a small open reading frame coding for delta hemolysin (*hld*) (Novick et al., 1993).

The four genes of the *agr* locus are highly conserved within the genus *Listeria* (Garmyn, Gal, Lemaitre, Hartmann & Piveteau, 2009). However, only one only self-inducible promoter precedes the *agrBDCA* operon in this organism. *In silico* analysis has failed to identify orthologs of the staphylococcal regulator RNAIII in Listeria or other Gram-positive bacteria (Wuster & Babu, 2008). The membrane-bound protein AgrB, processes the propeptide AgrD into a mature autoinducing peptide (AIP), which

is detected by the histidine kinase AgrC, inducing transcriptional regulation through activation of the regulator AgrA. In *L. monocytogenes*, the *agr* communication system is involved in adhesion to abiotic surfaces and early stages of biofilm formation (Riedel et al., 2009; Rieu, Weidmann, Garmyn, Piveteau & Guzzo, 2007), infection of the mammalian host (Autret, Raynaud, Dubail, Berche & Charbit, 2003), and soil adaptation (Vivant, Garmyn, Gal, Hartmann & Piveteau, 2015). Furthermore, a recent study showed an interconnection between the Agr system and the novel virulence regulator MouR. The dimeric GntR-family protein MouR positively regulates expression of the *agr* locus by binding to the operon promoter and regulating chitinase activity, biofilm formation, cell invasion and virulence in *L. monocytogenes* (Pinheiro et al., 2018).

There is interplay between the σ^B and agr systems of *S. aureus*. While σ^B increases *sar* expression, it apparently decreases RNA III production in a growth-phase dependent manner (Bischoff, Entenza & Giachino, 2001). However, although no direct link between *agr* and σ^B has been reported in *L. monocytogenes*, evidence of synergistic activity of the two systems has been reported. Several genes from the σ^B regulon showed significant changes in transcript levels between Δ*agrA* and WT strains at both saprophytic (25°C) and pathological (37°C) temperatures in *L. monocytogenes* EGD-e (Garmyn, Augagneur, Gal, Vivant & Piveteau, 2012). Interestingly, some of these genes are co-regulated by PrfA as well.

7.3.2. CtsR and HrcA

The class three stress gene regulator (CtsR) and the heat shock gene repressor (HrcA) have been associated with heat stress resistance in *L. monocytogenes* (Hu et al., 2007; Karatzas et al., 2003; Nair, Derre, Msadek, Gaillot & Berche, 2000). CtsR negatively regulates class III stress response genes (Nair, Derre, Msadek, Gaillot & Berche, 2000). Class III stress response genes are defined at those lacking the highly conserved CIRCE (controlling inverted repeat of chaperone expression) operator sequence (Hecker, Schumann & Volker, 1996) and whose induction by heat shock and general stress

conditions is σ^{B}-dependent. The expression of the Clp proteolytic system genes, including *clpP*, *clpE* and the *clpC* operon that includes *ctsR,* is negatively regulated by CtsR. The DNA binding activity of CtsR is regulated by McsAB-mediated phosphorylation, since phosphorylated CtsR is a substrate for degradation by the ClpCP complex (Kruger, Zuhlke, Witt, Ludwig & Hecker, 2001). Transcription of the modulators of CtsR repression (McsA and McsB) is an example of co-regulation by CtsR and σ^{B} (Hu et al., 2007). It results in a set of two promoter regions in the *ctsR-mcsA-mcsB-clpC* operon, a σ^{B}-dependent promoter upstream of *mcsA* and a σ^{A}-dependent promoter with a CtsR binding site upstream of *ctsR*. A total of 40 genes are co-regulated by σ^{B} and CtsR in *L. monocytogenes* adding to its stress resistance and virulence phenotypes (Hu et al., 2007; Karatzas et al., 2003). The role of the HrcA regulator was assessed and found to be involved in biofilm formation and disinfectant resistance (van der Veen & Abee, 2010). A σ^{B} consensus promoter sequence upstream of the 5′ portion of the *hrcA-grpE-dnaK* operon indicates a direct positive regulation of *hrcA* by σ^{B}, and provides evidence for a regulatory network involving these two regulators (Raengpradub, Wiedmann & Boor, 2008).

While σ^{B} positively regulates the transcription of class II stress response genes, both HrcA and CtsR negatively regulate class I and III heat-shock response genes, respectively. Several genes were found to be coregulated by either HrcA and CtsR, HrcA and σ^{B}, or all three regulators (Hu et al., 2007). Moreover, a total of 37 genes and 30 genes of the σ^{B} regulon were found coregulated by both CtsR and HrcA, respectively, though some of these were also found coregulated by at least one additional regulator. Overall, this intricate transcriptional network between σ^{B} and the negative regulators CtsR and HrcA is required to shape the heat shock response in *L. monocytogenes*.

7.3.3. CodY

Initially discovered in *B. subtilis* (Slack, Mueller & Sonenshein, 1993), the transcriptional repressor CodY is exclusive to low G+C content Gram-positive bacteria, including *L. monocytogenes* (Geiger &

Wolz, 2014). It is a GTP-binding protein that senses the intracellular GTP concentration as an indicator of nutritional limitations. Briefly, under starvation conditions the intracellular levels of GTP drop; this signal is sensed by CodY that thus can no longer bind to target DNA, and ceases the transcriptional repression of many genes required for stationary phase. Moreover, in *L. monocytogenes*, CodY can regulate carbon and nitrogen assimilation in response to both GTP and branched-chain amino acids (BCAA) (Bennett et al., 2007). Besides metabolism, CodY is recently known to regulate other cellular processes including stress resistance, motility and virulence in a highly versatile manner (Lobel & Herskovits, 2016).

The regulatory crosstalk between CodY and PrfA, AgrA and σ^B has also been studied. Under limited concentrations of BCAA, particularly isoleucine, CodY directly binds within the coding region of the master virulence regulator gene *prfA*, upregulating its transcription, thus triggering virulence in *L. monocytogenes* (Lobel, Sigal, Borovok, Ruppin & Herskovits, 2012). CodY activation of the two-component regulatory system Agr upon entry into stationary phase as also been shown (Bennett et al., 2007). In *S. aureus*, isoleucine limitation signals for de-repression of *agrA* as CodY no longer binds the regulatory region, leading to premature activation of *agr*, and consequently induction of the virulence state (Majerczyk et al., 2010; Pohl et al., 2009). Recently, it was found that CodY, directly and indirectly, represses σ^B, specifically under nutrient-rich conditions. CodY hierarchical regulation of stress-related genes was proposed when a CodY box was found upstream from *rsbV*, the first gene of the 4 gene operon that includes sigB (*rsbV, rsbW, sigB, rsbX*), thereby identifying a direct regulatory link between CodY and σ^B (Lobel & Herskovits, 2016). Thus, during mammalian infection, conditions in which BCAAs are considered to be limited (Brenner, Lobel, Borovok, Sigal & Herskovits, 2018; Lobel, Sigal, Borovok, Ruppin & Herskovits, 2012), CodY potentially regulates σ^B while it also promotes *prfA* transcription directly, via binding to the *prfA* gene, and indirectly, by relieving σ^B repression. In this regard, several virulence and stress resistance related genes indirectly repressed by CodY (e.g. *inlA*, *inlB*, *bsh* and *opuCA*), which were shown previously to be positively regulated by σ^B (McGann,

Raengpradub, Ivanek, Wiedmann & Boor, 2008), may be repressed in nutrient-rich conditions as a result of σ^B repression by CodY (Lobel & Herskovits, 2016).

7.3.4. MogR

The synthesis of flagella in *L. monocytogenes* is regulated by temperature, with higher expression at low temperature, and by the transcriptional repressor of all known flagellar genes, MogR (Shen & Higgins, 2006). MogR also contributes to the virulence since mutants lacking this regulator are attenuated by 250-fold in a BALB/c mouse model (Grundling, Burrack, Bouwer & Higgins, 2004). Two promoter regions were identified upstream of *mogR*, P1 and P2, sharing a common Rho-independent transcription termination (Toledo-Arana et al., 2009). P1 is a σ^B-dependent promoter, which includes a long 5'UTR overlapping with three genes on the opposite strand that are required for the synthesis of the flagellum, *lmo0675*, *lmo0676* and *lmo0677*. The P2 promoter produces the bicistronic mRNA *morgR-lmo0673*, which is constitutively expressed. However, the absence of the longer σ^B-dependent transcript is not sufficient to explain the fact that, at low temperatures, the Δ*sigB* mutant has increased motility, since a mutation that abolishes this transcript leads to increased flagellum gene expression not to increased motility (Toledo-Arana et al., 2009). Repression by MogR is less stringent at low temperatures to allow for flagella production and motility (Grundling, Burrack, Bouwer & Higgins, 2004). The bifunctional protein GmaR, a glycosyltransferase that has O-linked N-acetylglucosamine transferase activity for flagellin and also acts as a thermo-sensing anti-repressor that incorporates temperature signals into transcriptional control of flagellar motility (Kamp & Higgins, 2011). GmaR interacts directly with MogR and this interaction interferes with the capacity of MogR to bind DNA at promoters that contain the MogR operator sequence (Shen & Higgins, 2006). In the wild-type transcription of the *fliN-gmaR* operon is temperature dependent, with increased transcript levels produced at room temperature. It has been shown that GmaR is degraded in the absence of MogR and at 37°C, when the MogR:GmaR complex is less stable. Since MogR represses transcription of all flagellar motility genes, including the transcription of *gmaR*, temperature-

dependent changes in the stability of the MogR:GmaR anti-repression complex, due to conformational changes in GmaR, mediates repression or de-repression of flagellar motility genes in *L. monocytogenes* (Kamp & Higgins, 2011). Since motility genes have been shown to contribute to *L. monocytogenes* virulence, these data illustrate the complex contributions to virulence and regulatory networks involving σ^B.

7.3.5. PrfA

To conserve metabolic resources, *L. monocytogenes* uses temperature as a signal for sensing the host environment. Although generally not conserved, RNA thermometers are key transcriptional regulators commonly used by pathogenic bacteria for activating virulence genes (Ignatov & Johansson, 2017; Winkler & Breaker, 2003). In *L. monocytogenes*, the most well-known virulence RNA thermometer is located in the UTR preceding *prfA*. The translation of *prfA* is activated once the temperature rises to 37°C through the action of a RNA thermosensor. In its saprophytic state, *L. monocytogenes* encounters lower temperatures and the 5'-UTR of *prfA* mRNA forms a long hairpin structure, which partially masks the ribosome binding region, thereby blocking *prfA* translation and consequently the expression of virulence genes. Once in the host, the ambient temperature shifts to 37°C, which melts the hairpin structure and consequently activates *prfA* translation (Johansson et al., 2002). Additionally, *prfA* is down-regulated by a *trans*-acting riboswitch that responds to the S-adenosylmethionine (SAM) concentration during growth in the intestine. The *prfA trans*-regulation occurs by base-pairing with a prematurely terminated SAM riboswitch, causing transcriptional termination right after the transcription terminator hairpin. Since the SAM riboswitch interaction site is trapped in the hairpin structure, it is not able to inhibit *prfA* translation at low temperatures (Loh et al., 2009). The master virulence gene regulator PrfA regulates the genes comprising the virulence gene locus (*prfA-plcA-hly-mpl-actA-plcB*) in addition to other virulence genes located elsewhere in the chromosome of *L. monocytogenes*. The number of genes in the PrfA regulon varies substantially according to different studies and strains of *L. monocytogenes*, ranging from 10 (Scortti, Monzo,

Lacharme-Lora, Lewis & Vazquez-Boland, 2007) to 73 genes (Milohanic et al., 2003) in EGD-e, 112 genes in EGD (Marr et al., 2006) and 607 genes in 10403S (Ollinger, Bowen, Wiedmann, Boor & Bergholz, 2009).

The overlap between the σ^B and the PrfA regulons is perhaps one of the most well documented regulatory interconnections in *L. monocytogenes*. As discussed in section 6.2, the σ^B-dependent promoter region upstream *prfA* ($P2_{prfA}$) not only accounts for an increase of *prfA* transcription, but σ^B itself is also involved in reducing the cytotoxic effects of constitutively active PrfA, suggesting a multilevel regulatory link between σ^B and PrfA (Ollinger, Bowen, Wiedmann, Boor & Bergholz, 2009). The overlap between the σ^B and PrfA regulons has been well described (Chaturongakul et al., 2011; Milohanic et al., 2003; Ollinger, Bowen, Wiedmann, Boor & Bergholz, 2009). A search for hypothetical σ^B-dependent promoters among the PrfA regulon identified 22 putative promoter regions accounting for 33 genes in total, as some were organized in operons (Milohanic et al., 2003). It has been shown that PrfA positively regulates a core set of 12 genes preceded by a PrfA box and probably expressed from its σ^A-dependent promoter. However, a second set of PrfA-regulated genes lacking a PrfA box seem to be expressed from a σ^B-dependent promoter (Milohanic et al., 2003). However, only a total of 11 genes of the σ^B regulon were found to be coregulated by PrfA, from which some of these were even coregulated by at least one additional regulator (Chaturongakul et al., 2011).

Some virulence genes (e.g. *inlA*, *inlB* and *bsh*) are preceded by both PrfA boxes and σ^B promoters and appear to be coregulated by both PrfA and σ^B, even though contributions of σ^B and PrfA to *inlA* transcription may be apparent only under specific growth conditions (Kim, Marquis & Boor, 2005; McGann, Raengpradub, Ivanek, Wiedmann & Boor, 2008). During the transition from saprophytic to virulent state, *L. monocytogenes* relies upon complex regulatory networks that fine-tune the expression of virulence factors in response to environmental signals (Gray, Freitag & Boor, 2006). The master virulence gene regulator PrfA regulates the genes comprising the virulence gene locus (*prfA*-

plcA-hly-mpl-actA-plcB) in addition to other virulence genes located elsewhere in the chromosome of *L. monocytogenes*. While the full details of the complex regulatory interactions between these two regulons remain to be elucidated, it is clear that the interplay between them is a critical part of the decision making circuitry of this versatile pathogen.

7.3.6. Alternative sigma factors σ^C, σ^H, and σ^L

In addition to the housekeeping sigma factor σ^A and the general stress response regulator σ^B, the genome of *L. monocytogenes* has up to three additional alternative sigma factors (σ^C, σ^H, and σ^L) (Glaser et al., 2001). While σ^B is the primary regulator of the expression of general stress response genes, crucial in the survival of challenging environments (O'Byrne & Karatzas, 2008), homeostasis and resilience (Liu, Orsi, Boor, Wiedmann & Guariglia-Oropeza, 2017), the regulons controlled by other alternative sigma factors are comparatively smaller and less well defined. σ^C, which is specific to *L. monocytogenes* lineage II strains, has a small regulon (<10 genes) that contribute to heat stress resistance (Zhang, Nietfeldt, Zhang & Benson, 2005). The σ^H regulon (>150 genes) (Chaturongakul et al., 2011) is involved in intracellular growth, growth in minimal media and resistance to alkaline stress in *L. monocytogenes* (Rea, Gahan & Hill, 2004). The σ^L regulon (>70 genes) is involved in carbon and amino acid metabolism, as well as conferring resistance to stresses associated with various food preservation measures as low temperature, presence of salt, organic acids and the use of toxic compounds (Chan et al., 2008; Mattila et al., 2012; Raimann, Schmid, Stephan & Tasara, 2009; Tessema et al., 2012).

Several genes from multiple biological function categories were found to be coregulated by σ^B, and at least one other sigma factor (Mujahid et al., 2013). Of the σ^B regulon, (i) 92 genes were also regulated by σ^H; (ii) 31 genes by σ^L; and (iii) 2 genes by σ^C, all involved in a wide range of functional categories (e.g. energy metabolism, transport and binding). Moreover, some of these genes were found to be coregulated by at least one additional regulator producing a complex network of overlapping regulons

(Chaturongakul et al., 2011). The absence of phenotypic consequences in the loss of multiple sigma factors suggests functional redundancies among these regulators (Chaturongakul et al., 2011), which makes it futile to classify a gene as belonging to one specific regulon only.

8. Role of σ^B in saprophytism and food

This section will focus on the role of σ^B during the saprophytic lifecycle, in outdoor environments and within food production premises and foodstuff. Under these environmental conditions, *L. monocytogenes* may face a range of suboptimal conditions known to trigger Sigma B regulation as outlined in the previous sections.

8.1 Outdoor environments

Investigations into the role of σ^B during life in outdoor environments are scarce. In a commercial horticultural substrate (Supersoil, Scotts), deletion of *sigB* resulted in significantly lower populations of *L. monocytogenes* over a period of 4 weeks incubation in a climatic chamber with 11 h simulated days (20°C, 4 UV, and 3 luminescent lights), 13 h nights (16°C, no light), and 70% humidity (Gorski, Duhe & Flaherty, 2011). In another study, differential transcriptomic analysis using microarrays indicated that genes from the σ^B regulon were overrepresented in the set of genes with significant fold changes after 18h incubation in soil extracts at 25°C in the dark (Piveteau, Depret, Pivato, Garmyn & Hartmann, 2011). Upregulation of *sigB* transcription was observed after incubation in piggery lagoon effluent and a high proportion of the Sigma B regulon was differentially transcribed (Vivant, Desneux, Pourcher & Piveteau, 2017).

Soil may be a reservoir of *L. monocytogenes* from which transfer to plants may occur (NicAogain & O'Byrne, 2016; Vivant, Garmyn & Piveteau, 2013). This transfer process requires σ^B regulation as

transfer of *L. monocytogenes* from contaminated soil to radish was significantly lower when Sigma B was not functional (Gorski, Duhe & Flaherty, 2011). In another study, 5 h incubation on parsley leaves resulted in a significant reduction of the transcript levels of the σ^B-regulated genes *opuC* and *clpC* as well as *inlA*, *prfA* and *groEL* in comparison to the standard condition (TSB, 16 h, 25°C) (Rieu, Guzzo & Piveteau, 2010). Activity of σ^B is required for chitinase activity. Chitin, an insoluble polymer of N-acetyl-D-glucosamine which is degraded by two chitinases ChiA and ChiB, is abundant in the biosphere. In the absence of *sigB*, expression of both *chiA* and *chiB* was reduced (Larsen, Leisner & Ingmer, 2010). Furthermore, expression of *chiA* is σ^B-dependent (Toledo-Arana et al., 2009).

These results suggest that σ^B regulation is required for optimal fitness and survival in outdoors environments, though the factors which activate σ^B under these conditions remain to be deciphered. Clearly, *L. monocytogenes* could encounter many of the stress conditions described above (section 5) during life outdoors but the actual niche-specific environmental cues triggering σ^B activity have been poorly investigated to date.

8.2 Foodstuff and food production premises

Although *L. monocytogenes* is commonly found in the food chain (NicAogain & O'Byrne, 2016), reports on the activity of σ^B in complex food matrixes are scarce. In a study on the effect of NaCl content in liver pâté on the transcription of several target genes, transcription of *sigB* was observed in the three strains studied after 48 h incubation in pâté stored at 7°C, but the relative transcript level was significantly lower than in the standard BHI broth condition for two of the strains (Olesen, Thorsen & Jespersen, 2010). Reduction of the salt content from 3.66% to 2.42% (w/v water phase) or 1.39% (w/v) plus 0.241% (w/v) Ca-acetate and 1.461% (w/v) Ca-lactate did result in increased transcription of *sigB* for one of the three strains. In another study, levels of transcripts of *sigB* and some σ^B-regulated genes were similar when grown either on Ready-To-Eat turkey meat slices or BHI agar plates for 5 days at 15°C (Bae, Crowley & Wang, 2011). Transcription of *sigB* was quantified in Crescenza soft Italian cheese after

incubation at 4°C and 12°C for 24 h and 48 h. Higher levels were observed in cheeses stored at 12°C than in the laboratory condition (overnight BHI culture, 37°C) in two out of eleven strains of *L. monocytogenes* tested (Rantsiou, Mataragas, Alessandria & Cocolin, 2012). Data is available after incubation in fermented sausage, minced meat, soft cheese and ultra-High Temperature milk (Alessandria, Rantsiou, Dolci, Zeppa & Cocolin, 2013). The results suggest that transcript levels are strain dependent and that, for some strains, *sigB* transcription is increased in some food, for example minced meat, while storage temperature affected transcript levels in soft cheese and UHT milk; increased transcription was noticed at 12°C compared to 4°C but no general trend could be evidenced (Alessandria, Rantsiou, Dolci, Zeppa & Cocolin, 2013). Collectively these results suggest that in a complex environment such as a food matrix a multiplicity of factors, including the genetics and physiology of the specific strain being studied, can produce differences in the extent to which the general stress response is triggered.

In food processing facilities, *L. monocytogenes* faces many hostile environmental conditions. σ^B activity is required for maximum survival to surfactant stresses. Indeed, survival to quaternary ammonium compounds, benzalkonium chloride, cetylpyridinium chloride or sodium dodecyl sulphate (SDS) required activation of a functional Sigma B (Ryan, Gahan & Hill, 2008). The role of σ^B in the resistance to disinfectant was demonstrated under planktonic, static and continuous-flow biofilm conditions, challenged with benzalkonium chloride and peracetic acid. Indeed, survival of the *sigB* deletion mutant was lower than that of the wild type (van der Veen & Abee, 2010). The role of σ^B activity in desiccation was demonstrated experimentally under laboratory conditions when the nutrient content of a simulated food-contaminated surface was low (Huang, Ells & Hansen, 2015).

Resistance to antibiotics is emerging as a major health problem. The contribution of σ^B to the resistance to cephalosporin has been reviewed (Krawczyk-Balska & Markiewicz, 2016) and addition of vancomycin induces σ^B activity. Survival of *Listeria monocytogenes* to lethal concentrations of ampicillin, penicillin

G, vancomycin and to the bacteriocins nisin and lacticin 3147 require a functional σ^B (Begley, Hill & Ross, 2006; Shin, Brody & Price, 2010).

Food processing premises are environments that may favour the emergence of antimicrobial resistance through, for example, recurrent exposure to disinfectants, exposure to sub lethal stresses and horizontal gene transfer (Allen et al., 2016). Overall, these *in situ* experiments, either from outdoor niches, processing environments or foodstuff matrixes, suggest that a combination of factors and especially stresses drive physiological adaptation through many mechanisms. σ^B and σ^B-regulated genes appear to play a central role in this response to the environment.

9. Outstanding questions and future directions

Much has been learned about the role that σ^B plays in regulating the expression of specific protective mechanisms in *L. monocytogenes*. It is clear that it plays a pivotal role in allowing this versatile pathogen to cope with harsh environmental conditions, both inside and outside the host. However there is much that remains to be understood about the molecular mechanisms that lead to σ^B activation, and how its activity is modulated in response to different stress conditions. As discussed in this chapter, the stressosome is thought to be the principal hub for the integration of stress signals, however the mechanisms involved in transducing different signals through this organelle are largely unknown.

With the exception of blue light, where a good working model exists to account for signal detection and transduction by Lmo0799, even the nature of the stress signals detected is elusive. For example, while it is known that σ^B plays a crucial role in responding to acid and osmotic stresses (O'Byrne and Karatzas, 2008), the precise nature of the stress signals detected is unknown. In the case of acid

stress, one could hypothesise that local pH (i.e. local proton concentration) might be the crucial signal that is sensed but at present there is no evidence for this. Acidic conditions will have a plethora of effects on the cell including, *inter alia*, changes in metabolic fluxes, cellular energetics, protein folding, metal solubility, transporter activity, and it is conceivable that any of these effects could be sensed directly by the stressosome or indirectly through another sensory system that interacts with the stressosome. Likewise with osmotic stress, it is conceivable that the osmolyte itself could be detected (e.g. sodium in the case of NaCl stress) or some secondary effect of the osmolyte. For example NaCl stress typically triggers an influx of potassium to increase the internal osmotic pressure in order to counteract the water loss that arises when the external osmotic pressure increases (O'Byrne & Booth, 2002). So it is possible that potassium levels could be sensed by the stressosome, but equally other consequences of osmotic stress could also be sensed (e.g., reduced cell volume or effects on cellular energetics). Thus a fundamental issue that urgently needs to be addressed is the nature of the signals detected by the stressosome and whether each stress is separately detected by sensing some unique signal or whether more generic effects of stress on the cell (e.g. protein denaturation, or depleted energy pools) can be detected. The presence of multiple RsbR paralogues that differ in their N-terminal domains, the domains that form the protruding turrets from the stressosome (Marles-Wright et al., 2008) and that are proposed to be involved in signal sensing, suggests the capacity to sense multiple different signals but the evidence to support this idea is currently lacking.

In their recent study, Impens et al. (2017) discuss the possibility that the membrane-spanning miniprotein Prli42 might act to facilitate the transduction of signals to the stressosome from outside the cell. In particular they propose that Prli42 might play a role in detecting oxidative stress since there is an attenuated activation of σ^B in response to H_2O_2 exposure in a mutant lacking this miniprotein (Impens et al., 2017). The difficulty at this point is in devising a model that could account for such a sensory mechanism. Prli42 interacts with N-terminal domain of RsbR and this localizes the

stressosome to the membrane but it is difficult to see how such a small protein (31 amino acids) could transduce a signal from outside the cell to the stressosome, particularly as there are no extracellular residues predicted by the structural model (Impens et al., 2017). It is conceivable that Prli42 interacts with some other membrane protein whose role is to detect oxidative stress, and that this interaction is dependent on the presence of the stress signal. This interaction in turn could potentially influence the interaction of the Prli42 N-terminus with RsbR. Alternatively the role of Prli42 could simply be to localise the stressosome to the inner face of the membrane, so as to ensure that stress signals when they arise are detected at the earliest possible opportunity. However these ideas are highly speculative at present, and further work will be required to elucidate more fully the role of Prli42 in stress sensing and signal transduction. It will also be worth investigating whether other proteins can interact with the N-terminal domains of RsbR and its paralogues.

Although the stressosome structure has been partly resolved from *Bacillus subtilis* (Marles-Wright et al., 2008) and from the acetogen *Moorella thermoacetica* (Quin, Newman, Firbank, Lewis & Marles-Wright, 2008) many questions still remain about the structure, particularly about how the structure relates to its function, and how this changes when stress is applied. In *L. monocytogenes* the existence of the stressosome has now been shown biochemically (Impens et al., 2017), but very little structural information is available. So far structural studies have primarily focused on RsbR, RsbS, with little information about the structure when RsbT is present in the complex. Also the presence of the RsbR paralogues in the stressosome, and the overall stoichiometry when they are present has not been determined. The study by Impens et al. (2017) suggests that the RsbR paralogues Lmo0799, Lmo0161 and Lmo1642 are associated with the stressosome *in vivo*, as they co-precipitate with a tagged version of Prli42 in immunoprecipitation experiments. However their dynamic behavior (locations and abundance) in response to changing conditions has not yet been investigated. Nor is it known if they can form unique homodimer N-terminal turrets that protrude from the stressosome surface, nor

indeed whether they might be capable of heterodimer formation with other RsbR paralogues. Further structural studies using both X-ray diffraction crystallography and cryo-electron microscopy will be required to further elucidate the structural aspects while a combination of genetics and physiological studies will be needed to develop an understanding of how these structures relate to function *in vivo*.

Also uncertain at this point is the nature of σ^B regulation at a cellular level and how this regulation varies within populations. Most studies to date have used whole population-based approaches to investigate the regulation and regulatory effects of σ^B. Some evidence for heterogeneous activation of σ^B within a population comes from studies using a fluorescent σ^B reporter in cells subjected to osmotic shock. In one study, Utratna et al. (2012) showed that only a subset of the population activated σ^B in response to osmotic shock, although the proportion of cells with active σ^B increased as the magnitude of the stress was increased (Utratna, Shaw, Starr & O'Byrne, 2011). In *B. subtilis,* time lapse microscopy has been used to study cell-level activation of σ^B in response to energy stress and the results show that activation occurs in a pulsatile fashion, with the frequency of the pulses increasing in response to increased stress σ^B activity (Locke, Young, Fontes, Hernandez Jimenez & Elowitz, 2011). Stochasticity or “noise” was found to be an important component of this regulation. However a different experimental system using microfluidics to study individual cells found a different behavior in response to energy stress. In this case a single pulse of activation was observed, whose amplitude was dependent on the magnitude of the stress (Cabeen, Russell, Paulsson & Losick, 2017). The dynamics of activation is different when environmental stress is applied. In this case a single pulse of activation is observed, the amplitude of which is proportional to the rate at which the stress is applied (Young, Locke & Elowitz, 2013) or the magnitude of the stress (Cabeen, Russell, Paulsson & Losick, 2017), depending on the experimental system. Future work using these single-cell based approaches, combined with genetic approaches should prove very helpful in elucidating the

contribution of the different stressosome components to the activation of σ^B in response to different stress signals.

The nature of the regulatory links between σ^B and PrfA in *L. monocytogenes* also remains to be fully clarified. As discussed in this chapter several lines of evidence suggest that initiation of virulence within the host coincides with a switch between σ^B and PrfA as the organism progresses beyond the gastrointestinal tract (section 6.1; 7.3.5). There is significant overlap between the σ^B and PrfA regulons (Milohanic et al., 2003; Ollinger, Bowen, Wiedmann, Boor & Bergholz, 2009). The *prfA* gene is preceded by a σ^B promoter and the *inlAB* invasion genes are under σ^B control (Kim, Marquis & Boor, 2005; Kim, Gaidenko & Price, 2004). While CodY has been identified as a possible regulatory link between these systems (see section 7.3.3), it seems likely that the lifestyle switch that occurs between the survival mode (σ^B driven) and the virulence mode (PrfA driven) will be very carefully regulated and this inevitably means several layers of regulatory input. This research area is likely to receive a lot of future attention because targeting these regulatory mechanisms could potentially prevent the survival of this pathogen in the food chain and the gastrointestinal tract as well as blocking the activation of its virulence programme.

Over the decades fundamental research on this bacterium has had a huge impact on our understanding of how pathogens interact with the host. Indeed work on this microorganism has produced significant advances in cell biology, immunology as well molecular microbiology. The importance of *L. monocytogenes* as a food-borne pathogen both in terms of the challenge it presents to food producers and the risk to public health will ensure that it continues to be studied extensively for the foreseeable future. It seems likely that fundamental research into this pathogen will continue to pay dividends not only in terms new applications that will help control it but also in terms of our

understanding of fundamental aspects of biology, including gene regulation, mechanisms of sensory perception, cellular decision making processes, and host-pathogen interactions.

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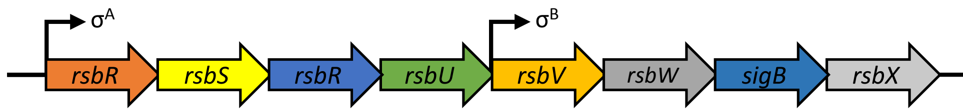
Fig. 1. Dorey et al. 2018

A



B

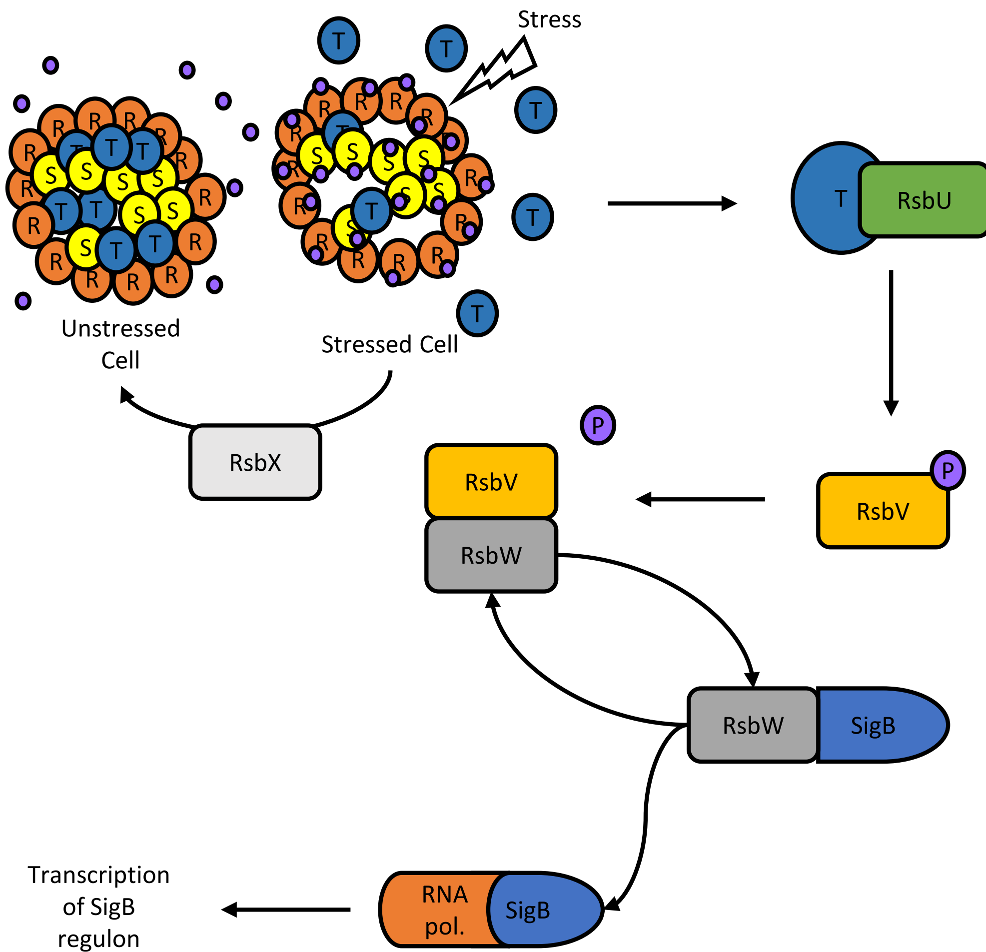


Table 1. Regulatory small RNAs of *Listeria monocytogenes* dependent on σ^B. This group includes sRNAs of all classes: three *trans*-acting sRNAs (SbrA, Rli47 and Rli33-1), two anti-sense RNAs (Anti-LhrC-5 and Anti2270) and a *cis*-acting regulatory element (Rli95). σ^B can also control the activity of the sRNA LhrA via the σ^B-dependent RNA-binding protein Hfq, while adding to stress tolerance and virulence in *L. monocytogenes*.

σ^B-dependent sRNAs	Targets	Associated Phenotypes and Remarks	References
Trans-acting sRNAs			
SbrA	Undetermined.	Undetermined; likely involved in the fine-tuning of gene expression and play a role in the σ^B-dependent regulation of stress response, metabolism, and virulence.	Nielsen et al., 2008; Oliver et al., 2009; Toledo-Arana et al., 2009.
Rli33-1	*oppA* (virulence-associated oligo-peptide binding protein), *lapB* (cell wall anchored virulence adhesion) and *tcsA (CD4+ T cell-stimulating antigen)* mRNAs.	Unlike its other sibling sRNAs, it is not induced by heme toxicity. Required for *L. monocytogenes* infection of macrophages. It has a role on bacterial pathogenicity.	Mraheil et al., 2011; Mollerup et al., 2016; dos Santos et al., 2018.
Rli47	Suggested targets: *lmo0636* and proteins Lmo0637 (methyltransferase) and Lmo2094 (L-fuculose-phosphate aldolase).	Highly expressed in cells on stationary phase, in macrophages, in the intestinal lumen, under oxidative stress and during soil adaptation on a Δ*agrA* mutant. Possible involvement in virulence and saprophytic lifestyle.	Oliver et al., 2009; Toledo-Arana et al., 2009; Mraheil et al., 2011; Mujahid et al., 2012; Vivant et al., 2015.
Cis-acting sRNAs			
Rli95	*lmo1884* (xanthine permease) and *lmo1885* (xanthine phosphoribosyl transferase).	Undetermined. Over-expressed in cells in macrophages. One of the two *Listeria* guanine riboswitches.	Mraheil et al., 2011; Krajewski et al., 2017.
Anti-sense sRNAs			
Anti2270	*lmo2270* (competence transcription factor ComK', N terminal).	Undetermined.	Mraheil et al., 2011; Wurtzel et al., 2012.
Anti-LhrC-5	*lhrC-5* (virulence associated sRNA) and *lmo0946* (hypothetical protein).	Over-expressed in hypoxia. Coding sequence overlaps the sequences of the sRNA LhrC-5 and *lmo0946.*	Toledo-Arana et al., 2009; Mraheil et al., 2011.
Hfq-dependent sRNAs			
LhrA	*lmo0302* (hypothetical protein), *chiA* (chitinase).	LhrA-mRNA duplex formation and stability is promoted by the σ^B-dependent RNA chaperone Hfq, as it also controls the translation and degradation of the target mRNAs *chiA* and *lmo0302.* Negative effect on the chitinolytic activity of *L. monocytogenes*.	Christiansen et al., 2006; Nielsen et al., 2011.