Overlaps in the regulatory networks of 
*Listeria monocytogenes* in response to 
environmental cues

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Directeur de thèse
“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”

Marie Skłodowska-Curie
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ABSTRACT

The facultative intracellular pathogen *Listeria monocytogenes* is a highly adaptable organism widely distributed in the environment. The ingestion of food contaminated with *L. monocytogenes* by at-risk individuals can ultimately lead to listeriosis, one of the leading causes of foodborne fatalities in developed countries. Regulatory networks are crucial for the adaptation and survival of this pathogen. The accessory gene regulator (Agr) system has been shown to be involved in virulence, biofilm formation, and survival of *L. monocytogenes*, affecting the transcription of over 700 genes. The alternative sigma factor Sigma B (σ^B_\text{B}) controls the general stress response in *L. monocytogenes*, regulating the transcription of almost 300 genes in response to stress. Earlier studies have suggested a regulatory overlap between the AgrA and the σ^B regulons in *L. monocytogenes*. While the functions of some regulatory systems are well understood, the biological role of most small regulatory RNAs (sRNAs) remains poorly characterized. This thesis aimed to decipher the interconnections between the AgrA and σ^B regulons in the regulatory network of *L. monocytogenes*, including the possible involvement of sRNAs.

Growth was observed in sterile soil and similar population dynamics were shown in the wild-type (WT) strain, ΔagrA and ΔsigB mutants. In biotic soil microcosms, viability of the WT strain declined steadily, underlining the challenging nature of live soil environments. The inactivation of the two systems simultaneously (ΔagrAΔsigB) further affected survival. Transcriptional analysis confirmed the expected effects of the mutations on known Agr- and σ^B-dependent genes. The ability to colonise the rhizosphere was also significantly compromised in the double mutant. Data highlighted the important role that these global regulatory systems play in the natural ecology of this pathogen.

A decrease in cell attachment was observed as a consequence of the Agr system inactivation. Using strains constructed to carry fluorescent reporters of either Agr or σ^B activity the spatiotemporal regulation of these systems was followed during biofilm formation. Deletion of the Agr system is beneficial for biofilm production under osmotic stress. Results also suggested a role for σ^B in biofilm formation that depended on the environmental conditions encountered. Delayed activation of agrA was shown in the ΔsigB background. Deletion of sigB
resulted in no activation of Agr in biofilm produced under osmotic stress. Overall, both Agr and σ^B systems were found to contribute to *L. monocytogenes* biofilm formation.

Using a combination of *in silico* and *in vivo* approaches, a binding interaction was predicted between the σ^B-dependent sRNA Rli47 and the Shine-Dalgarno region of the *ilvA* mRNA, which encodes threonine deaminase, an enzyme required for branched-chain amino acid biosynthesis. Both *ilvA* transcript levels and threonine deaminase activity were increased in the Δ*rli47* mutant. The Δ*rli47* mutant displayed a shorter growth lag in isoleucine-depleted media than the WT, and a similar phenotype was also observed in a mutant lacking σ^B*. RNA-seq analysis uncovered a significant role for Rli47 in modulating amino acid metabolism. The data point to a model where Rli47 is responsible for specifically repressing isoleucine biosynthesis as a way to restrict growth under harsh conditions, contributing to the survival of *L. monocytogenes* in niches both outside and within the mammalian host.

The diversity of global regulators as well as the crosstalk between the various regulatory systems in *L. monocytogenes* add complexity to its transcriptional networks under the most diverse environments. Understanding how individual stress signals are sensed and transcriptomic rearrangements achieved for a quick adaptation response might be a critical step in helping to develop strategies to prevent *L. monocytogenes* growth, survival, and dissemination.

**Keywords:** *Listeria monocytogenes*, adaptation, survival, Agr, σ^B*, transcriptional regulation, Rli47, RNA, sRNA, stress resistance, synergy, isoleucine, soil, rhizosphere, biofilm.
**RÉSUMÉ**

Listeria monocytogenes est une bactérie ubiquiste présente dans des habitats variés. Chez des personnes au système immunitaire affaibli, l’ingestion d’aliments contaminés par *L. monocytogenes* peut provoquer la listériose, une maladie qui se caractérise par un taux de mortalité très élevé. Sa capacité d’adaptation aux conditions environnementales repose sur un réseau complexe de régulations transcriptionnelle et post-transcriptionnelle. Le système de communication Agr participe à la régulation de plus de 700 gènes pendant la vie saprophyte et *in vivo* pendant le processus infectieux. Le facteur sigma alternatif Sigma B (σB) contrôle la réponse générale au stress et régule la transcription de près de 300 gènes. Certains gènes sont sous le contrôle direct ou indirect de ces deux régulateurs ce qui suggère une interconnexion entre le système Agr et σB. Par ailleurs, le rôle biologique de la plupart des petits ARN non-codants (ncARNs) reste peu caractérisé. L’objectif de ce travail de thèse était de décrypter les interconnexions entre les régulateurs AgrA et σB et d’étudier le rôle d’un ARN non codant qui pourrait participer à cette interconnexion.

Dans un sol stérilisé, les populations de *L. monocytogenes* augmentent et la délétion du gène *agrA* ou du gène *sigB* n’affecte pas leur croissance. Dans un sol non traité, la viabilité de la souche parentale diminue progressivement. Dans ces conditions, l’inactivation de l’un ou l’autre de ces systèmes a également affecté la survie. L’analyse transcriptionnelle a confirmé l’importance d’Agr et de σB pour la survie dans le sol. La capacité à coloniser la rhizosphère était également considérablement affectée par la délétion du gène *sigB*. Ces données illustrent le rôle que ces systèmes de régulation jouent dans l’écologie naturelle de ce pathogène.

rencontrées. La structuration du biofilm variait d’un empilement uniforme de cellules dans un milieu riche, à des structures filamentueuses et dispersées en conditions de stress osmotique. Ces résultats confirment la contribution des systèmes Agr et σB au cours de la croissance sessile de *L. monocytogenes*.

Le rôle de l’ARN non codant Rli47 a été étudié par des approches *in silico* et *in vivo*. Plusieurs cibles ont été identifiées dont *ilvA* qui code la thréonine désaminase, une enzyme de la voie de biosynthèse des acides aminés à chaîne ramifiée. L’interaction entre Rli47 et la région Shine-Dalgarno de l’ARNm *ilvA* a été confirmée expérimentalement. La délétion de *rli47* provoque une augmentation des taux de transcrits *ilvA* ainsi que de l’activité de l’enzyme. Par ailleurs, dans un milieu appauvri en isoleucine, le temps de latence du mutant Δ*rli47* est réduit par rapport à celui de la souche parentale. Ce phénotype a également été observé dans un mutant dépourvu de σB. L’analyse du transcriptome confirme le rôle important de Rli47 dans la modulation du métabolisme des acides aminés. Les données amènent à un modèle théorique dans lequel Rli47 est un répresseur de la voie de biosynthèse de l’isoleucine ce qui pourrait limiter la croissance de *L. monocytogenes* lorsque les conditions environnementales sont défavorables.

L’élucidation de ces réseaux de régulation permettra de mieux comprendre l’écologie de *L. monocytogenes* et pourrait permettre le développement d’alternatives innovantes visant à limiter son développement.

**Mots-clés:** *Listeria monocytogenes*, adaptation, survie, Agr, σB, réseaux de régulation, Rli47, ARN, ncARNs, résistance au stress, synergie, isoleucine, sol, rhizosphère, biofilm.
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>*OH</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>%</td>
<td>Percent</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>&lt;</td>
<td>Smaller than</td>
</tr>
<tr>
<td>=</td>
<td>Equal</td>
</tr>
<tr>
<td>&gt;</td>
<td>Over than</td>
</tr>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>ActA</td>
<td>Actin-polymerizing surface protein</td>
</tr>
<tr>
<td>ADI</td>
<td>Arginine deiminase</td>
</tr>
<tr>
<td>Agr</td>
<td>Accessory gene regulator</td>
</tr>
<tr>
<td>AIP</td>
<td>Autoinducing peptide</td>
</tr>
<tr>
<td>ALS</td>
<td>Acetolactate synthase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>asRNA</td>
<td>Antisense sRNA</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine tri-phosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>Acid tolerance response</td>
</tr>
</tbody>
</table>

*B. subtilis*  
*Bacillus subtilis*

<table>
<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>BCAA</td>
<td>Branched-chain amino acid</td>
</tr>
<tr>
<td>BCAT</td>
<td>Branched-chain aminotransferase</td>
</tr>
<tr>
<td>BCFA</td>
<td>Branched-chain fatty acids</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain-heart infusion</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>CC</td>
<td>Clonal complex</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>Chi</td>
<td>Chitinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>CIRCE</td>
<td>Controlling inverted repeat of chaperone expression</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CodY</td>
<td>Transcriptional repressor...</td>
</tr>
<tr>
<td>ComX</td>
<td>Competence pheromone precursor</td>
</tr>
<tr>
<td>CtsR</td>
<td>Class three stress gene regulator</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DHAD</td>
<td>Dihydroxy-acid dehydratase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DS</td>
<td>Diffusion sensing</td>
</tr>
<tr>
<td>E. faecalis</td>
<td><em>Enterococcus faecalis</em></td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>eDNA</td>
<td>Extracellular DNA</td>
</tr>
<tr>
<td>EEA</td>
<td>European economic area</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EPS</td>
<td>Extracellular polymeric substance</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ES</td>
<td>Efficiency sensing</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>GABA</td>
<td><em>Gamma</em>-Aminobutyric acid</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamate decarboxylase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>Gln</td>
<td>Glutamine</td>
</tr>
<tr>
<td>GmaR</td>
<td>Glycosyltransferase, thermo-sensing anti-repressor</td>
</tr>
<tr>
<td>GSR</td>
<td>General stress response</td>
</tr>
<tr>
<td>GW</td>
<td>Glycine-tryptophan</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>H2O</td>
<td>Water</td>
</tr>
<tr>
<td>H2O2</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>H2S</td>
<td>Hydrogen sulphide</td>
</tr>
<tr>
<td>Hfq</td>
<td>RNA-binding chaperone</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HGFR</td>
<td>Hepatocyte growth factor receptor</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>HrcA</td>
<td>Heat shock gene repressor</td>
</tr>
<tr>
<td>Ile</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>Inl</td>
<td>Internalin</td>
</tr>
<tr>
<td>IPMDH</td>
<td>Isopropylmalate dehydrogenase</td>
</tr>
<tr>
<td>IPMI</td>
<td>Isopropylmalate isomerase</td>
</tr>
<tr>
<td>IPMS</td>
<td>Isopropylmalate synthase</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobases</td>
</tr>
<tr>
<td>Ketol-acid isomeroeductase</td>
<td>KARI</td>
</tr>
</tbody>
</table>

- L. *aquatica*  
- L. *booriae*  
- L. *cornellensis*  
- L. *fleishmannii*  
- L. *floridensis*  
- L. *grandensis*  
- L. *grayi*  
- L. *innocua*  
- L. *ivanovii*  
- L. *marthii*  
- L. *monocytogenes*  
- L. *newyorkensis*  
- L. *plantarum*
<table>
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<tr>
<td>L. riparia</td>
<td><em>Listeria riparia</em></td>
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<tr>
<td>L. rocourtiae</td>
<td><em>Listeria rocourtiae</em></td>
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<tr>
<td>L. seeligeri</td>
<td><em>Listeria seeligeri</em></td>
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<tr>
<td>L. weihonstephanensis</td>
<td><em>Listeria weihonstephanensis</em></td>
</tr>
<tr>
<td>L. welshimeri</td>
<td><em>Listeria welshimeri</em></td>
</tr>
<tr>
<td>Lap</td>
<td>Lipopolysaccharide assembly protein</td>
</tr>
<tr>
<td>Leu</td>
<td>Leucine</td>
</tr>
<tr>
<td>LGI</td>
<td>Listerial genomic island</td>
</tr>
<tr>
<td>LIPI</td>
<td><em>Listeria</em> pathogenicity island</td>
</tr>
<tr>
<td>LLO</td>
<td>Listeriosin O</td>
</tr>
<tr>
<td>LOV</td>
<td>Light, oxygen, voltage</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine-rich repeat</td>
</tr>
<tr>
<td>Met</td>
<td>Methionine</td>
</tr>
<tr>
<td>MLST</td>
<td>Multilocus sequencing typing</td>
</tr>
<tr>
<td>MogR</td>
<td>Transcriptional repressor</td>
</tr>
<tr>
<td>MouR</td>
<td>Virulence regulator</td>
</tr>
<tr>
<td>Mpl</td>
<td>Metalloprotease</td>
</tr>
<tr>
<td>MprF</td>
<td>Lysylphosphotidylglycerol modifier</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>n</td>
<td>Number</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NCBI</td>
<td>National center for biotechnology information</td>
</tr>
<tr>
<td>ncRNA</td>
<td>Non-coding RNA</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Superoxide</td>
</tr>
<tr>
<td>OpuC</td>
<td>Carnitine transporter system</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
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P  Promoter
PALCAM  Polymyxin-acriflavin-lithium-chloride-ceftazidime-aesculin-mannitol
PDI  Photodynamic inactivation
PEP  Phosphoenolpyruvate
PI-3K  Phosphatidyl inositol 3 kinase
Plc  Phospholipase
PMF  Proton motive force
pPplA  Peptide pheromone
PrfA  Positive regulatory factor A
PTS  Phosphotransferase system
PycA  Pyruvate carboxylase A

QS  Quorum sensing

RBS  Ribosome binding site
RNA  Ribonucleic acid
RNA-seq  RNA sequencing
RNase  Ribonuclease
ROS  Reactive oxygen species
rRNA  Ribosomal RNA
Rsb  Regulator of Sigma B
RT-PCR  Reverse transcriptase polymerase chain reaction

*S. aureus*  *Staphylococcus aureus*
SAM  S-adenosylmethionine
Sar  Staphylococcal accessory gene regulator
Sbr  Sigma B-dependent RNA
SD  Shine-Dalgarno
*S. enterica*  *Salmonella enterica*
*S. epidermidis*  *Staphylococcus epidermidis*
SNP  Single Nucleotide Polymorphism
Sps  Signal peptidase
sRNA  Small RNA
ST  Sequence type
STAS  Sulphate transporter and anti-sigma factor antagonist
T  Thymine
TD  Threonine deaminase
Trp  Tryptophan
TSB  Tryptone soy broth
U  Uracil
UHT  Ultra-heat treatment
USA  United States of America
UTR  Untranslated region
UV  Ultraviolet
Val  Valine
WGS  Whole Genome Sequencing
WT  Wild-type
$\sigma^{A/B/C/F/H/L}$  Sigma factor A/B/C/F/H/L
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Marinho C. M., Gal L., Garmyn D., Piveteau P. and O’Byrne C. Activation of Agr and σB systems during Listeria monocytogenes EGD-e biofilm development suggests regulatory overlap. (In preparation)

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Section 1.4.3 and 1.5 have recently been published in the first author book chapter “Role
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1.1. *Listeria monocytogenes*

*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 *et al.*, 2011; Radoshevich and 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 and Chikindas, 2007; NicAogain and O’Byrne, 2016; O’Byrne and Karatzas, 2008; van Schaik and Abee, 2005). The latter properties make this foodborne 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 and 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 it 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.

1.1.1. **General Characteristics and Taxonomy**

The foodborne pathogen *Listeria monocytogenes* is a small (0.5 μm in diameter and 1 to 2 μm in length), Gram-positive, rod-shaped, non-spore forming, facultatively anaerobic bacterium that is motile at temperatures below 37°C (Farber and Peterkin, 1991; Mead *et al.*, 1999; Rocourt and Buchrieser, 2007). Peritrichous flagella confer its motility at 20°C to 25°C,
however at 37°C the production of flagellin is greatly reduced (Peel et al., 1988).

Known today as *Listeria monocytogenes*, the first report of this bacterium dates from 1921, isolated from the cerebrospinal fluid of a soldier who died from meningitis in 1918 (Dumont and Cotoni, 1921). This new bacterial species was later characterized by Murray and co-workers in 1926 from infected laboratory guinea pigs and rabbits. Because of the increased number of monocytes that were found in the bloodstream of infected animals, the organism was originally named *Bacterium monocytogenes* (Murray et al., 1926). The first human isolate dates from 1929 (Nyfeldt, 1929), and its first connection to human disease was suspected quite early (Burn, 1936) as well; this bacterium remained recognized as a veterinary pathogen for years. Later, it was changed to *Listerella hepatolytica* before assuming its current nomenclature in 1940 by James Hunter Harvey Pirie (Pirie, 1940). The first reported outbreak of human listeriosis was in New Zealand in 1979 (Carbonnelle et al., 1979). In 1983, the bacterium was officially recognised as a foodborne pathogen (Schlech et al., 1983). Despite a significant trend of increased confirmed invasive listeriosis cases since 2006, fewer than round 2,300 cases per year were registered in the EU/EEA over the last 3 years, where the annual case fatality rates range between 12.7 and 20.5% (EFSA, 2017). In the United States of America, similar numbers were reported, with an estimated 1,600 people get listeriosis each year, and about 260 deaths (CDC, 2018). The most recently confirmed substantial listeriosis outbreak occurred in South Africa, 978 cases clinically confirmed with a fatality rate of 27% (WHO, 2018). Although the number of cases of listeriosis is small, the high rate of death associated with this infection makes it a significant public health concern (WHO, 2018).

From a physiological point of view, *L. monocytogenes* is a psychrotolerant bacterium, able to grow at temperatures as low as -0.1°C (Walker et al., 1990), and whose optimal growth temperature ranges between 30 and 37°C (Seeliger and Jones, 1986), tolerating the presence of 40% NaCl (Liu et al., 2005). Its optimal growth pH is close to neutrality, although *L. monocytogenes* is able to grow at pH between 4.6 and 9 (De Vos et al., 2009). *L. monocytogenes* is a facultative anaerobic, catalase positive and oxidase negative bacterium. The biochemical identification of *L. monocytogenes* is based on the following characteristics: hydrolysis of esculin, presence of alpha-mannosidase, fermentation of D-arabitol, L-rhamnose and methyl-alpha D-glucopyranoside, but no fermentation of D-xylose, D-ribose, glucose-1-phosphate and D-tagatose. In addition, *L. monocytogenes* does not produce indole or
hydrogen sulphide (H$_2$S) and does not contain nitrate reductase, urease or gelatinase. Its isolation can be done through selective media such as Polymyxin-Acriflavin-Lithium-Chloride-Ceftazidime-Aesculin-Mannitol Agar (PALCAM) medium. On PALCAM medium, the hydrolysis of esculin by Listeria spp. results in the formation of a black halo around olive-green colonies, which makes it possible to differentiate L. monocytogenes and other Listeria species from the majority of other microorganisms.

1.1.2. **Phylogeny and Genomics**

Phylogenetic classification of Listeria was mostly based on analyses of the 16S rRNA sequences, rather than on phenotypic data. Within the family Listeriaceae, the genus Listeria is very close to Brochothrix, and both genera are positioned between Lactobacillus and Bacillus, belonging to the large, systematic phylum of Firmicutes (De Vos et al., 2009).

The Listeria genus has been greatly expanded in the last decade and currently contains 20 species (Figure 1.1), including the established species L. innocua, L. monocytogenes, L. welshimeri, L. seeligeri, L. ivanovii, L. grayi, L. marthii, L. roucourtiae, L. fleischmannii, L. floridensis, L. aquatica, L. newyorkensis, L. cornellensis, L. weihenstephanensis, L. grandensis, L. riparia, L. booriae and the more recently identified species L. thailandensis, L. costaricensis and L. goaensis (Bertsch et al., 2013; Boerlin et al., 1991; den Bakker et al., 2014; Doijad et al., 2018; Graves et al., 2010; Lang Halter et al., 2013; Leclercq et al., 2010; Leclercq et al., 2019; Núñez-Montero et al., 2018; Orsi and Wiedmann, 2016; Weller et al., 2015a). The only pathogenic Listeria species are L. monocytogenes, which is the causative agent of foodborne listeriosis in humans and a source of infection in a number of other host species, and L. ivanovii, which affects animals, mainly sheep and cattle, with few reported cases of infections in humans (Cummins et al., 1994; Lessing et al., 1994; Snapir et al., 2006).
Figure 1.1. Phylogenetic tree of *Listeria* species. Maximum likelihood phylogeny based on concatenated amino acid sequences of 325 single copy core genes present in all *Listeria* species. *Listeria* species belonging to clade I and clade II are designated. The bar indicates the number of inferred nucleotide substitutions per site. Adapted from Leclercq et al., 2019.

The species of the genus *Listeria* can also be grouped into two distinct clades based on their provenance (Figure 1.1). Clade I, the *Listeria sensu stricto* group, includes the only two pathogenic strains *L. monocytogenes* and *L. ivanovii* (Seeliger, 1984), and four commensal strains *L. marthii* (Graves et al., 2010), *L. innocua*, *L. welshimeri*, and *L. seeligeri* (Chiara et al., 2015). Among other sources, all clade I strains have been isolated from gastrointestinal tract samples or from animal origin foodstuffs, suggesting a specific interaction of these species with the hosts (Dahshan et al., 2016; Huang et al., 2007; Nayak et al., 2015; Schardt et al., 2017; Wang et al., 2017). Clade II, the *Listeria sensu lato* group, contains the species *L. fleischmanni* (Bertsch et al., 2013), *L. weihenstephanensis* (Lang Halter et al., 2013), *L. rocourtiae* (Leclercq et al., 2010), *L. aquatica*, *L. cornellensis*, *L. riparia*, *L. floridensis*, *L. grandensis* (den Bakker et al., 2014), *L. grayi*, *L. newyorkensis*, *L. booriae* (Weller et al., 2015a), *L. costaricensis* (Núñez-Montero et al., 2018), *L. goaensis* (Doijad et al., 2018) and *L. thailandensis* (Leclercq et al., 2019).
2019), which have been isolated from outdoors environments or food-associated surfaces. Genomic analyses showed that none of the *Listeria sensu lato* species harbour the *Listeria* pathogenicity island 1, which includes the major virulence genes *prfA, plcA, hly, mpl, actA, plcB*, or the *Listeria* pathogenicity island 2, which includes the internalin genes *inlA* and *inlB*. With the exception of *L. grayi*, none of the other *sensu lato* species carry the motility genes that encode flagellar proteins. Phylogenetic analyses indicate that the ancestor of *Listeria sensu stricto* and *L. grayi* acquired the flagellar biosynthetic genes through horizontal gene transfer from an ancestor of *Bacillus cereus* (Chiara et al., 2015). The only gene related to motility present in *sensu lato* species is *mogR*; this gene encodes for the motility gene repressor MogR, which specific function in this species remains to be determined (den Bakker et al., 2014).

Genomics of *Listeria sensu lato* species showed an underrepresentation of genes associated with internalin domains (Chiara et al., 2015; den Bakker et al., 2010), suggesting that expansion of internalin genes happened after the divergence between *Listeria sensu stricto* and *Listeria sensu lato*. Considering that internalins have been shown to play a central role in the interactions of *L. monocytogenes* and mammalian hosts, it further supports that *sensu lato* strains are unlikely to be pathogenic for mammalian species (Pizarro-Cerda et al., 2012). However, the genome of *L. fleischmannii* subsp. *Coloradonensis* was found to harbour the putative mosquitoical toxin MTX1 from *Bacillus*, suggesting that some *sensu lato* strains may be able to cause disease in insects (den Bakker et al., 2013).

A total of 13 serotypes of *L. monocytogenes* were established based on somatic and flagellar antigens, and these are further divided into four lineages (I, II, III and IV) at present (Lomonaco et al., 2015; Orsi et al., 2011; Piffaretti et al., 1989; Rasmussen et al., 1995; Wiedmann et al., 1997). Lineage I contains serotypes 1/2b, 3b, 4b, 4d and 4e; whilst Lineage II includes 1/2a, 1/2c, 3a, and 3c (Orsi et al., 2011; Piffaretti et al., 1989). Lineage I and II are mostly associated with clinical (sporadic and outbreak cases) and food isolates. Lineage III, which consists of 4a and 4c along with some 4b strains, was thought to contain three distinct groups, IIIA, IIIB and IIIC but recently IIIB has been renamed as the fourth lineage (Liu et al., 2006; Rasmussen et al., 1995; Roberts et al., 2006; Ward et al., 2008). Lineage III and IV strains are far less isolated and appear to be most associated with ruminants and non-primate animals (Orsi et al., 2011; Wiedmann et al., 1997). The most widely used lab strains EGD-e, EGD and 10403S are all
serovar 1/2a. Approximately 95% of all food or clinical isolates belong to 1/2a, 1/2b, or 4b serotypes (Farber and Peterkin, 1991; Swaminathan and Gerner-Smidt, 2007; Tappero et al., 1995), with 1/2a being most commonly found in food and 4b causing disease most often (Glaser et al., 2001).

Individual isolates of *L. monocytogenes* exhibit clear differences in terms of their ability to cause human infection. Over the years, several phylogenetic studies have been evaluating relationships between *L. monocytogenes* strains, identifying not only the four lineages but also clonal groups that correlate with infection and/or source (Bergholz et al., 2018). Clonal complexes (CC) were defined as groups of sequence types (STs) differing by only a single nucleotide polymorphism from another member of the group (Ragon et al., 2008). Primarily, using multilocus sequencing typing (MLST), strains were discriminated into twenty-four CC grouping isolates, that had a recent common ancestor (Ragon et al., 2008). Another pioneer phylogeny study on three hundred *L. monocytogenes* isolates collected from different sources in 42 countries and 5 continents concluded that CC1, CC2 and CC3 were highly prevalent in lineage I, while CC9 and CC7 were the most frequent in lineage II (Chenal-Francisque et al., 2011). Subsequent studies have led to the identification of additional CCs, though for some lineages, such as lineages I and II, few new CCs were found, suggesting that most of the clinically relevant CCs have been identified (Haase et al., 2014). A total of 63 CCs has been identified to date (Radoshevich and Cossart, 2018). Moreover, Hasse et al. reported that most of the CCs (72%) in their *L. monocytogenes* isolate collection, were not specific for any single source, implying that human infections did originate from the transmission of a saprophyte from the environment to farms and infected livestock and eventually to food products, the actual source of infection to the consumer (Haase et al., 2014).

A global worldwide distribution of the most frequent clones was observed with predominance of CC1 and CC2 (except in northern Africa for CC1), CC9 ranked third in Europe and the Western Hemisphere, while CC3 ranked among the 4 most common clones in all regions (Chenal-Francisque et al., 2011). Recently, a metaanalysis of the relative frequency of CCs before and since 2000, including ten studies on isolates with broad geographic provenance, concluded that the frequency of detection of historically dominant CCs, such as CC1 and CC2, has recently decreased, while the frequency of previously uncommon CCs such as CC5, CC6, CC9, and CC121 is on the rise (Bergholz et al., 2018). However, geographical differences in
dominant CCs are expected as not only environmental factors but also dietary choices are likely to drive survival and transmission of specific *L. monocytogenes* genotypes (Bergholz et al., 2018). For example, back in the mid-1950s, CC101 was frequently isolated from human disease in Europe and North America, it then became less frequent until more recently when it expanded again, relative to other CCs (Haase et al., 2014). Conversely, this recent expansion of CC101 was not corroborated by more recent studies from Australia (Jennison et al., 2017) and Europe (Bertrand et al., 2016). Similarly, CC121, which is the most frequent clone among food isolates, appears to be expanding in Australia as well as Europe, whereas incidence of clinical isolates of CC1 and CC3 showed no sign of decline in Australia (Jennison et al., 2017).

Many of these CCs have been linked to epidemic clones responsible for multiple listeriosis outbreaks, e.g. CC1 comprised reference strains of the 1981 Nova Scotia coleslaw outbreak, the 1983-1987 Switzerland Vacherin Mont d’Or outbreak, the 1986-1987 California soft cheese outbreak, and the 1992 French pork tongue in jelly outbreak; and CC2 included reference strains of the 1983 Massachusetts pasteurized milk outbreak, the 1987-1988 United Kingdom and Ireland pâté outbreak, and the 1997 Italy gastroenteritis outbreak (Cantinelli et al., 2013; Chenal-Francisque et al., 2011). It suggested that these isolates may represent genotypes of increased fitness for host infection. Determination of dominant CCs over time may help understanding genomic characteristics underlying fitness. It can as well be indicative of alterations in selective pressures that may lead to changes in CCs predominance. Genetic elements associated with virulence phenotypes have been identified in some clones of *L. monocytogenes*. Among other genetic differences, an ~ 8 kb DNA fragment named *Listeria* pathogenicity island 4 (LIPI-4) containing genes that are involved in carbohydrate metabolism was identified in strains belonging to CC4, one of the most virulent CCs involved in meningitis cases (Maury et al., 2016), although the involvement of LIPI-4 in other listeriosis cases remains to be determined (Bergholz et al., 2018). Similarly, certain CCs of *L. monocytogenes* harbour genome traits that underlie the ability to adapt to stresses associated to the food processing environment, thus facilitating its survival and transmission in the food chain. Interestingly, in Canada, outbreak and sporadic listeriosis isolates predominantly belong to CC8. These isolates harbour the genomic island LGI1 (Gilmour et al., 2010; Knabel et al., 2012; Kovacevic et al., 2016) that carries *emrE*-encoded small multidrug-resistant efflux pump associated with increased tolerance to quaternary ammonium
compounds, broad spectrum antibacterial agents most commonly used in the food processing industry (Kovacevic et al., 2016).

Environmental adaptations of *L. monocytogenes* are also associated with resistance to heavy metals, most commonly cadmium and arsenic (McLauchlin et al., 2004). Moreover, it was shown that the same plasmid that carries the genetic elements responsible for benzalkonium chloride tolerance (*bcrABC*) and cadmium efflux (*cadA2*) can be carried in the same plasmid (Katharios-Lanwermeyer et al., 2012). Despite its presence in the chromosome of strains belonging to CC1, CC2, and CC6 complexes (Lee et al., 2013), *cadAC*, coding a cadmium efflux system, repressed virulence of *L. monocytogenes* in the *Galleria mellonella* insect model (Parsons et al., 2017), leaving unknown its role in human listeriosis. The 35-kb chromosomal *Listerial genomic island 2* (LGI2) is widely present in CC1, CC2 and CC4 strains. It contains arsenic resistance genes (*arsA1* and *arsA2*) and confers mobility to the heavy metal resistance genes into different locations of the chromosome of *L. monocytogenes* (Lee et al., 2013), and its presence suggests a role in virulence in this pathogen (Lee et al., 2017).

*L. monocytogenes* is capable of survival and growth in a wide range of food-related stresses including refrigeration temperatures, food preservatives and several salts including NaCl that are frequently used to control bacterial growth in food products. A list of strains from various CCs, primarily CC2 and CC11, showed greater salt tolerance (Hingston et al., 2017). Strains belonging to CC6 and CC7 had significantly higher innate resistance to the bacteriocin nisin compared to strains belonging to CC2, CC3, CC5, CC9, and CC11 (Malekmohammadi et al., 2017). Adaptation of *L. monocytogenes* to either low pH or osmotic stress increased resistance to nisin, limiting its effectiveness as a food preservative (Bergholz et al., 2013; Bonnet and Montville, 2005; van Schaik et al., 1999).

In 2001, the first complete genomes of *L. monocytogenes* EGD-e (serovar 1/2a) and *L. innocua* CLIP 1262 (serovar 6a) were released (Glaser et al., 2001). *L. monocytogenes* EGD-e genome has a size of 2,944,528 base-pairs, a G+C content of 38% and 2,853 protein-coding genes. Genome analysis revealed a large number of predicted genes encoding surface and secreted proteins, transporters, and transcriptional regulators, consistent with the ability of this species to adapt to diverse environments (Glaser et al., 2001). Complete genome sequences of over 80 strains of the genus *Listeria* are now available on the website Listeriomics.
Chapter 1

(https://listeriomics.pasteur.fr/Listeriomics/#bacnet.Listeria), a total of 171 complete genome assemblies of *Listeria* strains can be accessed on NCBI genomes at the moment. The main difference between *L. monocytogenes* and non-pathogenic *L. innocua* is the absence of most of the virulence gene cluster, including the positive regulatory factor A (PrfA), the master virulence regulator (Chakraborty *et al.*, 2000; Glaser *et al.*, 2001). Evidence suggest that ancestral strains of *Listeria* spp. gained the virulence gene cluster, which was later lost by *L. innocua* (Vazquez-Boland *et al.*, 2001). Despite the presence of homologues of the main virulence gene cluster, *L. seeligeri* is recognised as non-pathogenic as it rarely causes infection in humans or animals (Gouin *et al.*, 1994; Rocourt *et al.*, 1987). Soil has been suggested as the major habitat of ancestral *Listeria*, where the virulence gene cluster was needed primarily against soil protozoa and then evolved to facilitate intracellular survival and lifecycle (Chakraborty *et al.*, 2000; Ly and Muller, 1990). Notwithstanding, genomic comparisons point to a model where the progenitor species contained the virulence gene cluster, which was eventually lost by non-pathogenic *Listeria* (Vazquez-Boland *et al.*, 2001).

1.1.3. **Metabolism and Growth Requirements**

**i. Carbon Source**

Besides utilising glucose as a preferred carbon source, *L. monocytogenes* is also able to utilise a number of other sugars including fructose, mannose, trehalose, cellobiose, maltose and glycerol (Premaratne *et al.*, 1991; Tsai and Hodgson, 2003), or even ferment galactose, lactose and sucrose (Seeliger and Jones, 1986). However, various other sugars including, melibiose, raffinose, sorbose, sorbitol, mannitol, arabinose and ribose do not support growth (Premaratne *et al.*, 1991). Organic acids including acetate, pyruvate, lactate, citrate, succinate, ketoglutarate, malate and fumarate do not support the growth of *L. monocytogenes* (Premaratne *et al.*, 1991). Moreover, *L. monocytogenes* is able to utilize glucose 6-phosphate (glucose-6P) which is transported by the phosphate antiporter UhpT, encoded by a gene (*uhpT*) under the control of PrfA (Chico-Calero *et al.*, 2002; de las Heras *et
al., 2011). *Listeria* harbours the aromatic amino acid biosynthesis genes (*aro*) that are responsible for producing the electron acceptor menaquinone, and displays an NADH oxidase activity (Glaser et al., 2001; Patchett et al., 1991; Stritzker et al., 2004). Moreover, a recent study suggested that energy production in Gram-positive bacteria might also be derived from an anaerobic respiration allowed by extracellular electron transfer, which transfers the electrons released by the respiratory chain to extracellular electron acceptors, instead of oxygen (which is usually reduced to H₂O on the cytosolic side of the membrane) (Light et al., 2018). Two glucose transport systems are present in *L. monocytogenes*, a high affinity proton phosphoenolpyruvate-dependent phosphotransferase system (PTS) and a low-affinity proton motive force (PMF)-mediated system (Parker and Hutkins, 1997).

Based on genome analysis *L. monocytogenes* possesses complete glycolytic and pentose-phosphate pathways (Glaser et al., 2001). Glucose is metabolised aerobically via the pentose phosphate pathway until α-ketoglutarate; however, the next step cannot be achieved because the tricarboxylic acid cycle is incomplete (Eisenreich et al., 2006). *L. monocytogenes* lacks the genes encoding enzymes that convert α-ketoglutarate to succinyl co-A (2-ketoglutarate dehydrogenase and succinyl co-A synthetase), succinyl co-A to succinate (succinate dehydrogenase) and also malate to oxaloacetate (malate dehydrogenase) (Glaser et al., 2001; Trivett and Meyer, 1971). However, succinate can be produced from gamma aminobutyrate via the intermediate succinyl semialdehyde (Feehily et al., 2013). The incomplete cycle results in a reduced capacity of *L. monocytogenes* to generate ATP by these means, which suggests that this pathway is primarily used for biosynthesis and not for energy production (Trivett and Meyer, 1971). Oxaloacetate is hence thought to be derived from the carboxylation of PEP-derived pyruvate by pyruvate carboxylase, resulting in a slowdown of branched-chain amino acid (BCAAs) biosynthesis (Eisenreich et al., 2006). As the by-products of glucose metabolism, both aerobically and anaerobically, are lactic acid and acetic acid, it is expected that *L. monocytogenes* derives energy primarily by substrate level phosphorylation during glycolysis and the subsequent generation of a proton motive force (PMF), similar to other lactic acid bacteria (Lungu et al., 2010; Pine et al., 1989; Salema et al., 1996).

In regards to carbon metabolism, the formation of oxaloacetate by intracellular *L. monocytogenes* depends fully on the carboxylation of pyruvate catalysed by pyruvate carboxylase (PycA) (Schar et al., 2010). Pyruvate carboxylase has been shown to be extremely
important in virulence, specifically during replication in mammalian cells (Schar et al., 2010). The pentose phosphate pathway and not glycolysis is the primary pathway for carbohydrate metabolism and it appears that *L. monocytogenes* switches to utilising phosphorylated glucose and glycerol as carbon sources when inside the host cell (Joseph et al., 2006). While nitrogen metabolism inside host cells is not fully understood, ethanolamine, ammonium and arginine are expected sources as opposed to glutamine (Joseph et al., 2006; Schauer et al., 2010). Taken together with a knowledge of intracellular carbon sources, it appears that *L. monocytogenes* avoids competition with the host cell for both glucose and glutamine (Hain et al., 2007).

### ii. Vitamins and Iron

The biosynthetic pathways for riboflavin, thiamine, biotin and thioctic acid are missing from the *L. monocytogenes* genome (Glaser et al., 2001), which is consistent with the fact that these four vitamins are essential for growth previously observed by Welshimer (1963). Iron is a vital element for nearly all living organisms, due to the fact that its involved in biological processes such as biosynthesis of DNA, energy production and regulation of gene expression, and its role as a cofactor for numerous cellular enzymes (Lechowicz & Krawczyk-Balska, 2015). Although iron is one of the four most important and most abundant compounds on Earth’s crust, its low solubility of ferric form predominant in the aerobic environment limits the availability of this microelement in the environment for living organisms (Andrews et al., 2003). Inside mammalian hosts, iron is mostly complexed to the heme subunit of hemoglobin, or bound to proteins such as ferritin, lactoferrin, and transferrin (Hentze et al., 2004). The ability of *L. monocytogenes* to acquire and utilise iron is not essential during infection but could also support its growth and survival in many diverse environmental niches (McLaughlin et al., 2011). As a way to limit infection, the host organism developed an iron sequestration mechanism to inhibit bacterial growth through a phenomenon called nutritional immunity (Weinberg, 1974; Latunde-Dada, 2009). It has led intracellular bacteria to overcome these iron-dependent host immune responses through the activation of diverse mechanisms that allow iron acquisition from the iron-binding proteins in the host. *L. monocytogenes* is capable of utilizing host iron sources such as heme and hemoglobin via
HupDGC, as well as dopamine and norepinephrine (Jin et al., 2006; Weinberg, 2009). To utilise heme as an iron source, L. monocytogenes firstly lyses the erythrocytes, binds hemoglobin, extracts heme molecules, and then imports them for intracellular degradation, liberating free iron (Choby & Skaar, 2016). Heme can be an essential source of iron for several invading bacteria; however, its acquisition must be tightly regulated as excess heme has a growth-inhibitory effect on L. monocytogenes. The LhrC1-5 family of small non-coding RNAs, together with the two-component system LisRK, play a role in the adaptation of L. monocytogenes to heme stress conditions (dos Santos et al., 2018).

It has been suggested that L. monocytogenes can assess its location through iron sensing, as iron-limitation results in the increased expression of PrfA-regulated virulence factors listerioliysin (LLO) and ActA for phagosomal escape, movement in host cytosol and cell-to-cell spread, and increased iron concentrations result in the upregulation of internalin proteins (InlA and InlB) required for invasion (Lungu et al., 2009; Gray et al., 2006; Conte et al., 1996; Bockmann et al., 1996; Conte et al., 2000). In Listeria, iron homeostasis is controlled by the regulatory protein Fur (ferric uptake regulator) or a functional equivalent (Andrews et al., 2003). In the presence of sufficient levels of iron, Fur acts as a repressor in that a Fur–iron complex prevents gene transcription by binding to a specific Fur-box sequence (Escolar et al., 1999). A total of 14 genes were identified as part of the Fur regulon in L. monocytogenes, including genes encoding ferrous iron transporters, ferrichrome ABC transporters, and proteins involved in iron storage (Ledala et al., 2010). In L. monocytogenes, there is an increase in fur transcription under iron limiting conditions, which is autoregulated in vivo (Ledala et al., 2007). The disruption of the fur gene led to a decrease in the virulence potential of L. monocytogenes, however this defect could be reverted by overloading the host with iron (Rea et al., 2004). Furthermore, the expression of fur is negatively regulated by PerR, a Fur homolog involved in the oxidative stress response of L. monocytogenes (Rea et al., 2005). Therefore, it is yet unclear whether the virulence defect of the L. monocytogenes Fur mutant results from deregulation of iron uptake systems or is due to a more direct involvement in virulence gene regulation (McLaughlin et al., 2011).

Iron storage does also influence the host-pathogen relationship. Similar to the host, bacteria use ferritin-like proteins for the storage of this metal, but recent findings show the host can target these storage proteins as part of the humoral immune response. The L. monocytogenes genome encloses a ferritin encoding gene, designated fri, which is a member of the Fur-
regulon and considered necessary for full listerial infection (Ledala et al., 2010; Glaser et al., 2001; Dussurget et al., 2005). Deletion of the fri gene results in an increased sensitivity to oxidative stress, potentially due to a decreased capacity to store iron, a reduced ability to proliferate inside macrophages, and a decrease in the expression of virulence factors such as LLO (Dussurget et al., 2005; Olsen et al., 2005). In addition to its role in virulence, the ferritin protein in L. monocytogenes was found to be the principal cold shock protein (Hebraud & Guzzo, 2000), necessary for heat and cold shock resistance (Dussurget et al., 2005), which could in turn contribute to Listeria survival in food matrices that are subject to temperature variations.

**iii. NITROGEN AND BRANCHED-CHAIN AMINO ACIDS METABOLISM**

There is conflicting evidence regarding amino acid requirements of L. monocytogenes. Leucine (Leu), isoleucine (Ile), valine (Val), methionine (Met), arginine (Arg), cysteine (Cys), glutamine (Gln), histidine (His) and tryptophan (Trp) were shown to be essential for growth (Welshimer, 1963), although a more recent study has shown that histidine and tryptophan are not required (Premaratne et al., 1991). Since all amino acid biosynthesis pathways are present in the L. monocytogenes genome (Glaser et al., 2001), the requirement for amino acids may be due to the repression of certain amino acid biosynthetic pathways under certain growth conditions.

The BCAAs (isoleucine, leucine and valine) are small nonpolar amino acids with aliphatic side chains. BCAAs are often found in different protein structures due to small differences in size, hydrophobicity, degree and position of branching of the side chains (Brosnan and Brosnan, 2006). L-leucine is found primarily in α-helices, loops and leucine zippers, while L-isoleucine and L-valine are predominantly in β-sheets. Bacteria, fungi and plants synthesize BCAAs through a conserved pathway. Despite being essential for mammalian growth, the biosynthetic pathways for Ile, Leu and Val biosynthesis are missing in mammals (Amorim Franco and Blanchard, 2017).

Moreover, the BCAA biosynthetic pathway also provides intermediates required for the synthesis of vitamin B5 and branched-chain fatty acids (BCFAs) (Kaiser and Heinrichs, 2018; Webb et al., 2004). BCFAs are the predominant fatty acids in Gram-positive bacterial
membranes that determine its biophysical properties (Kaneda, 1991), thus contributing to bacterial adaptation to changes in temperature, pH, salinity, and CO₂ (Annous et al., 1997; Chihib et al., 2003; Giotis et al., 2007; Jones et al., 1997; Jydegaard-Axelsen et al., 2004; Klein et al., 1999; Mastronicolis et al., 2006; Nichols et al., 2002). In total, BCFAs comprise 75 to 98% of L. monocytogenes membrane, and the lack of BCFAs have been shown to increased susceptibility to antimicrobials and lysozyme digestion though decreasing production of the virulence factor listeriolyisin O and consequently intracellular growth and virulence (Annous et al., 1997; Chihib et al., 2003; Jydegaard-Axelsen et al., 2004; Sen et al., 2015; Sun and O’Riordan, 2010; Sun et al., 2000; Zhu et al., 2005).

In L. monocytogenes, most of the BCAA biosynthesis genes are encoded in one operon consisting of nine genes (ilvDBHC-leuABCD-ilvA), named the ilv-leu operon. Compared to other pathways that require many enzymes to synthesize a single amino acid (e.g., nine enzymes are required for the conversion of L-aspartate to L-lysine), the BCAA biosynthetic pathway includes only eight enzymes for the synthesis of all three BCAAs (Amorim Franco and Blanchard, 2017). Moreover, four out of the eight enzymes are shared for the biosynthesis of all three BCAA (acetolactate synthase, ketol-acid reductase, dihydroxy-acid dehydratase and branched-chain amino acid aminotransferase); three are only responsible for the synthesis of L-leucine (2-isopropylmalate synthase, isopropylmalate isomerase, 3-isopropylmalate dehydrogenase); and solely one is exclusively involved in L-isoleucine biosynthesis (threonine deaminase) (Figure 1.2).

The first step in the biosynthesis of L-isoleucine is catalysed by the ilvA-encoded threonine deaminase (TD) (Figure 1.2). TD was one of the first enzymes in which metabolic control via negative feedback was observed (Umbarger, 1956; Umbarger and Brown, 1956, 1958); the presence of the end product, L-isoleucine, in the growth medium reduces TD activity in Escherichia coli. Allosteric regulation was also observed in TD (Eisenstein, 1994). This enzyme has two separate binding sites, site A, where L-threonine binds to the catalytic site and the site B, which can bind to either L-threonine or L-isoleucine (Changeux, 1961). Whenever L-threonine binds to site A, the catalytic activity of TD is activated and increased if L-threonine also binds to site B. However, when L-isoleucine binds to site B, the affinity of the enzyme for L-threonine at site A decreases and TD activity is diminished. The presence of the end products impacts the velocity of the reaction in a L-threonine concentration manner. L-
isoleucine acts as an allosteric inhibitor and L-valine as an allosteric activator of TD (Changeux, 1961; Eisenstein, 1991; Monod et al., 1965). Overall, the homotropic cooperativity profile of TD is explained by the greater affinity of the substrates and analogues for the regulatory sites than for the catalytic sites (Eisenstein, 1995), suggesting that the allosteric changes observed from the low- to high-activity state occur synchronously and progressively throughout the range of L-threonine concentrations. Although the impact of the deletion of the ilvA gene in L. monocytogenes remains to be determined, in B. subtilis it generates isoleucine auxotrophy (Rosenberg et al., 2016).

Three genes (ilvB, ilvN and alsS) encode for acetalactate synthase (ALS), an enzyme catalysing the second enzymatic step of L-isoleucine pathway and it is shared in the biosynthesis all three BCAAs (Figure 1.2). It catalyses the synthesis of (S)-2-acetolactate or (S)-2-aceto-2-hydroxybutanoate from the decarboxylation of pyruvate and its condensation with either pyruvate (L-leucine and L-valine common pathway branch) or 2-ketobutyrate (L-isoleucine pathway branch) (EC 2.2.1.6). The next step is catalysed by the ilvC-encoded ketol-acid isomeroreductase (KARI). This enzyme converts the products of ALS, (S)-2-acetolactate or (S)-2-aceto-2-hydroxybutanoate, to (2R)-2,3-dihydroxy-3-methylbutanoate or (R)-2,3-dihydroxy-3-methylpentanoate, respectively, using NADPH (reduced nicotinamide adenine dinucleotide phosphate) as a reductant (E.C. 1.1.1.86) (Figure 1.2). Equally, the ilvD-encoded dihydroxyacid dehydratase (DHAD) is an enzyme required for the synthesis of all three BCAAs. DHAS is responsible for the synthesis of 3-methyl-2-oxobutanoate and (S)-3-ethyl-2-oxopentanoate from (2R)-2,3-dihydroxy-3-methylbutanoate or (R)-2,3-dihydroxy-3-methylpentanoate, respectively (E.C. 4.2.1.9).

Three enzymes are exclusively required for L-leucine biosynthesis (Figure 1.2). The leuA-encoded isopropylmalate synthase (IPMS) catalyses the conversion of 3-methyl-2-oxobutanoate and acetyl-CoA to (2S)-2-isopropylmalate and CoA, respectively (E.C. 2.3.3.13). The second exclusive enzyme in the branch of L-leucine biosynthesis is the leuCD-encoded isopropylmalate isomerase (IPMI), which catalyses the isomerization of (2S)-2-isopropylmalate to (2R,3S)-3-isopropylmalate (E.C. 4.2.1.33). The leuB-encoded isopropylmalate dehydrogenase (IPMDH) is responsible for converting 3-isopropylmalate to 2-ketoisocaproate via oxidation of the second alcohol and decarboxylation on a NAD⁺-dependent manner (E.C. 1.1.1.85).
The final step in the synthesis of all three BCAAs is catalysed by the *lm*0978-encoded branched-chain aminotransferase (BCAT) (Figure 1.2). This enzyme is involved in the transfer of the α-amino group of L-glutamate to the α-carbon of 4-methyl-2-oxopentanoate, 3-methyl-2-oxobutanoate, and (S)-3-methyl-2-oxopentanoate, the keto acid precursors of L-leucine, L-valine, and L-isoleucine, respectively (E.C. 2.6.1.42).

![Diagram of the BCAA biosynthesis pathway](https://biocyc.org/LMON169963/NEW-IMAGE?type=PATHWAY&object=BRANCHED-CHAIN-AA-SYN-PWY&detail-level=2)

**Figure 1.2. Listeria monocytogenes** EGD-e pathway of branched chain amino acid biosynthesis. Genes and encoded enzymes are shown in blue. Adapted from MetaCyc (https://biocyc.org/LMON169963/NEW-IMAGE?type=PATHWAY&object=BRANCHED-CHAIN-AA-SYN-PWY&detail-level=2).

---

2 pyruvate

\[ \text{CO}_2 \quad \text{H}^+ \]

Acetolactate synthase, *ilvB*

Acetolactate synthase small subunit, *ilvW*

Acetolactate synthase, *alaS*

(S)-2-acetolactate

NADPH + H^+

Ketol-acid reductoisomerase, *ilvC*

\[ \text{H}_2\text{O} \]

(2R)-2,3-dyhydroxy-3-methylbutanoate

Dihydroxy-acid dehydratase, *ilvD*

3-methyl-2-oxobutanoate

acetyl-CoA + H_2O

coenzyme A + H^+

2-isopropylmalate synthase, *leuA*

L-glutamate

2-oxoglutarate

BCAA aminotransferase, *lm*0978

(2S)-2-isopropylmalate

H_2O

Isopropylmalate isomerase, *leuC* *leuD*

2-isopropylmaleate

\[ \text{H}_2\text{O} \]

(2R,3S)-3-isopropylmalate

NAD^+

3-isopropylmalate dehydrogenase, *leuB*

\[ \text{NADH} + \text{H}^+ \]

(2S)-2-isopropyl-3-oxosuccinate

Spontaneous

\[ \text{H}^+ \quad \text{CO}_2 \]

4-methyl-2-oxopentanoate

L-glutamate

2-oxoglutarate

BCAA aminotransferase, *lm*0978

L-leucine

L-valine
Intracellular pathogens face the challenge of direct competition with the host for intracellular BCAAs since they are essential nutrients in humans. Concentrations of BCAAs have been estimated in some host environments relevant to pathogens such as bloodstream, where BCAA levels range from 20 - 92 µM for Ile, 40 - 250 µM for Leu, and 65 - 266 µM for Val (Wishart et al., 2018). Moreover, several intracellular pathogens, including *L. monocytogenes*, might rely on BCAA biosynthesis for replication inside host cells as both BCAA biosynthesis and transport systems have been shown to promote virulence during infection, suggesting that pathogens encounter BCAA limitation *in vivo* (Atkins et al., 2002; Awasthy et al., 2009; Bange et al., 1996; Joseph et al., 2006; Lobel and Herskovits, 2016; Lobel et al., 2012). Indeed, Ile availability influences virulence gene expression in this pathogen (Brenner et al., 2018; Lobel et al., 2012). Despite encoding all BCAA biosynthesis genes (Figure 1.2), under laboratory conditions *L. monocytogenes* synthesizes very little Ile, Leu, and Val as it still...
requires BCAAs supplementation to support optimal growth under nutrient limiting conditions, behaving as a partial auxotroph (Eisenreich et al., 2006; Hain et al., 2007; Joseph and Goebel, 2007; Premaratne et al., 1991; Tsai and Hodgson, 2003). Two possible explanations have been suggested for this paradox (Kaiser and Heinrichs, 2018). L. monocytogenes carboxylates pyruvate to form oxaloacetate in environments where glucose is the sole carbon source and shortage of this indirect BCAA biosynthetic precursor might explain the limited amounts of BCAAs synthesized (Eisenreich et al., 2006; Joseph and Goebel, 2007). Another possible factor could be tight transcriptional repression of the biosynthetic operon (Brenner et al., 2018).

Additionally, BCAAs are effectors of the global transcriptional regulator CodY in Gram-positive bacteria, a conserved transcription regulator in Firmicutes that senses and promotes adaptation to nutrient starvation (Guedon et al., 2001; Ratnayake-Lecamwasam et al., 2001; Tani et al., 2002). Although all three BCAAs bind to CodY and activate its DNA-binding activity, binding of Ile induces a stronger effect on CodY activity as, among all BCAAs, depletion of this amino acid is the sole to relieve repression of CodY-regulated genes in L. monocytogenes (den Hengst et al., 2005; Guedon et al., 2005; Handke et al., 2008; Kaiser et al., 2018; Kim and Burne, 2017; Lobel et al., 2012; Santiago et al., 2013; Shivers and Sonenshein, 2004; Villapakkam et al., 2009).

1.1.4. BIOFILM FORMATION AS A SURVIVAL AND PERSISTENCE STRATEGY

Biofilms are structured communities of bacterial cells embedded in a self-produced matrix of extracellular polymeric substances (EPSs) often composed of polysaccharides, that are responsible for adhesion to surfaces and cohesion (Donlan, 2002; Flemming et al., 2007). The extracellular matrix is an important component of L. monocytogenes biofilms (Colagiorgi et al., 2017). Exopolysaccharides, proteins, and eDNA are the main molecules composing the biofilm matrix of several bacteria (Flemming et al., 2007). EPSs confer several features to biofilm, such as the structure complexity, resistance to removal and destruction, and an increased resistance to antimicrobials. Biofilm formation can be viewed as five distinct steps: attachment, cell-cell adhesion, proliferation, maturation and dispersion (Figure 1.3). In the
last stage of biofilm development, *L. monocytogenes* cells are able to detach from the biofilm and to disperse into the environment in their planktonic form, representing a potential source of contamination (Reis-Teixeira *et al.*, 2017).

**Figure 1.3. Representation of the biofilm developmental stages.** (a) Attachment, the first step involves planktonic cells reversible attachment to surfaces; (b) Cell-cell adhesion, the adhered cells begin to form a monolayer and to produce extracellular matrix; (c) Proliferation, the cells within the self-produced extrapolymeric matrix continue to grow and form multilayer microcolonies; (d) Maturation, cells are irreversibly attached to the surface and embedded in the matrix forming the mature biofilm; (e) Dispersion, in the last stage of biofilm formation, cells are able to detach from the biofilm, returning to planktonic form and disperse, ready to colonize new surfaces. Adapted from Colagiorgi *et al.* 2017.

Biofilm formation by *L. monocytogenes* is influenced by a multitude of conditions such as resource availability, temperature, the nature of the attaching surface and the presence of other microorganisms. A study that assessed the role of growth medium and temperature in *L. monocytogenes* biofilm formation, reported that there was a significant impact on biofilm formation of growth medium, but only a minimal effect of growth temperature (Moltz and Martin, 2005). *L. monocytogenes* is able to form biofilm within a wide range of temperatures (4 to 42°C), although higher amounts of biofilm are formed at *Listeria* optimal growth temperature 37 °C than at 4°C (Di Bonaventura *et al.*, 2008; Tomićić *et al.*, 2016). However,
at cold temperatures, higher levels are formed on glass compared to the more hydrophobic stainless steel and polystyrene (Di Bonaventura et al., 2008). It has been suggested that biofilm formation at low temperatures could be regulated by genes that are not implicated in this process at higher temperatures (Piercey et al., 2016). Although lineage II strains have been shown to form higher levels of biofilm (Borucki et al., 2003), a strong variability in biofilm forming ability has been observed, casting doubt on the correlation between lineages and biofilm forming ability (Di Bonaventura et al., 2008).

Several studies reported a limited sensitivity to biocides of *L. monocytogenes* within biofilm compared to its planktonic counterpart (Aarnisalo et al., 2007; Chavant et al., 2004; Gram et al., 2006; Pan et al., 2006; Robbins et al., 2005). Moreover, the ability of bacteria to form biofilm seems to enhance the resistance of this microorganism to antimicrobials (Pan et al., 2006; van der Veen and Abeel, 2011). Although many antimicrobials used in food industry for removing *L. monocytogenes* are usually able to reduce and inactivate the microorganisms, there are still some risks related to the detachment and the regrowth of the cells (Minei et al., 2008; Poimenidou et al., 2009).
1.2. **ECOLOGICAL NICHES AND TRANSMISSION ROUTES**

*L. monocytogenes* is a ubiquitous pathogen found widely in the natural environment. In addition to the great diversity of habitats that *L. monocytogenes* can colonize, this bacterium has the ability to persist for long periods of time. Moreover, multiple pathways of transmission between distinct environments ranging from soil to the cytosol of host cells add complexity on the ecology of *L. monocytogenes* (Figure 1.4).

![Diagram of ecological niches and transmission routes](image)

**Figure 1.4.** Factors affecting the survival and transmission of *Listeria monocytogenes* in the environment and food chain. Survival of *L. monocytogenes* in the soil is influenced by factors such as the composition of the soil, the competing microbiota, weather events, irrigation from contaminated sources, as well as human and animal faecal contamination. Consequently, crops can be contaminated...
with this pathogen at the point of harvest, which may introduce the pathogen into the food processing environment. However, the agricultural produce can become contaminated while being processed if adequate cleaning and decontamination practices are not in place. Contaminated ready-to-eat food represent a particular risk to the consumer, especially those that are immunocompromised. Taken from NicAogáin et al., 2016 with permission.

1.2.1. ADAPTATION OF L. MONOCYTOGENES TO NON–HOST ASSOCIATED NICHES

Soil plays a central role in the transfer of *L. monocytogenes* from food to the human or animal host. Some practices such as agricultural recycling of organic waste products (sewage sludge, slurry and composts) or irrigation with water of poor microbiological quality could be pathways for entry of pathogens into the soil (Figure 1.4). The application of sewage sludge to agricultural land could result in the intake of $10^6$ to $10^8$ *L. monocytogenes* per hectare per year (Garrec et al., 2003; Paillard et al., 2005). Animals may also represent a source of *L. monocytogenes* into the soil (Figure 1.4) due to the large amount of asymptomatic carriage of this pathogen by livestock (Wesley, 1999). Splashing caused by rain or watering is a possibility of transfer of soil to crop production (Figure 1.4). In the literature, such a transfer has, for example, been shown for a strain of *L. innocua* to the aerial parts of parsley (Girardin et al., 2005) and to salad leaves (Oliveira et al., 2011).

i. Soil

Over the years, a considerable number of studies reported soil as a natural reservoir of *L. monocytogenes*, with the first reports demonstrating the presence of *L. monocytogenes* in soil dating back to the 1960s and 1970s (Welshimer, 1960; Welshimer and Donker-Voet, 1971). However, more recent studies have suggested that soil contamination by the organism may come from other sources such as sewage, animal manure and decaying plant vegetation (Figure 1.4) (Fenlon et al., 1996). Studies reported an increased prevalence of *L.
monocytogenes in non-cultivated soils rather than in cultivated ones (Dowe et al., 1997; Weis and Seeliger, 1975). Soil populations of L. monocytogenes are generally low; the prevalence of L. monocytogenes throughout the French territory was estimated less than $10^4$ per gram of dry soil (Locatelli et al., 2013a).

L. monocytogenes can persist in soil over a period of time ranging from less than a week to several months depending on several abiotic factors such as the type of soil, water content, pH and temperature (Ivanek et al., 2009; Locatelli et al., 2013b; McLaughlin et al., 2011). Competition by different microflora for nutrients, soil chemical properties as well as geographical and meteorological conditions are factors that may affect its survival (Ivanek et al., 2009; McLaughlin et al., 2011; Strawn et al., 2013). Survival of L. monocytogenes is higher in fine soil with high clay content, and the cation content is more compatible with long term survival of this pathogen (Locatelli et al., 2013b). Soil microbiota is another feature that can highly affect the survival of L. monocytogenes. Interactions between L. monocytogenes and different types of protozoa have previously been demonstrated (Ly and Muller, 1990; Pushkareva and Ermolaeva, 2010; Zhou et al., 2007). Sterilisation of soil can lead to an increase in growth of L. monocytogenes suggesting that the microbiota of the soil, including bacteriophage and protozoa, have an effect on persistence of the bacterium (McLaughlin et al., 2011; Vivant et al., 2017; Vivant et al., 2015; Vivant et al., 2014; Vivant et al., 2013b).

Spatial factors such as proximity to urban areas, agricultural farms or water sources can lead to higher detection of L. monocytogenes (Sauders et al., 2012; Strawn et al., 2013; Weller et al., 2015c). The fact that incidence of L. monocytogenes is much higher in samples taken from farms compared to an undeveloped area, suggests that the presence of humans and animals is highly associated with isolation of this pathogen (Chapin et al., 2014). Similarly, in the United States, the presence of L. monocytogenes was detected in 1.6% of the soils sampled, while the prevalence was much higher in the urban habitat where 11.6% of the soils were positive for L. monocytogenes (Sauders et al., 2012). In France, the presence of L. monocytogenes has also been demonstrated in grassland soils with an incidence of 17% (Locatelli et al., 2013a). Overall, dissemination of L. monocytogenes from the soil could be a key step in propagation cycle of the bacteria, as the presence of L. monocytogenes in soil generates a risk of transfer of the bacterium to vegetation and crop production.
ii. **Farm Environment, Crops and Livestock**

*L. monocytogenes* was detected in the agricultural environment in silage, with 2.5% to 22.2% of the silage samples collected in the agricultural environment containing *L. monocytogenes* (Fenlon, 1985). A more recent study reported 38% of silage samples taken daily from a dairy farm were positive for *L. monocytogenes* (Ho et al., 2007). To a lesser extent, *L. monocytogenes* was also detected in 2.3% and 2.6% of silages made from maize and hay, respectively (Pauly and Tham, 2003). *L. monocytogenes* can survive for several months, when the anaerobic conditions are not respected and/or the pH of the silage is not low enough (> 4).

Cabbage, salads (Jamali and Radmehr, 2013; Uzeh and Adepoju, 2013), melons (Laksanalamai et al., 2012) and maize (de Valk et al., 2005) have, for example, already been identified as carriers of *L. monocytogenes*. In addition, a literature review detailing the incidence of *L. monocytogenes* in vegetables showed that soybean sprouts, cabbages, cucumbers, potatoes, radishes and tomatoes may be possible carriers of *L. monocytogenes* (Beuchat, 1996). Populations of *L. monocytogenes* on carrot, watercress, lettuce, radish, spinach and tomato seeds did not internalize within seedlings but did persist on the surface of plants throughout the cultivation period between $10^5$ and $10^6$ CFU per g of soil for 9 days after germination (Jablason et al., 2005). In this study, *L. monocytogenes* was also detected on the leaves of young plants but no internalization of the bacteria was observed. After inoculation of barley shoots, the roots and plants were colonized with *L. monocytogenes*, but again no internalization was noted (Kutter et al., 2006). Vegetables, such as carrots and radishes, kept in direct contact with the soil throughout their development, are therefore a category of products potentially exposed to *L. monocytogenes*. After 3 months of development in soil artificially contaminated with *L. monocytogenes*, 50% of the radishes were positive for the pathogen (Van Renterghem et al., 1991). However, contamination is not systematic since *L. monocytogenes* was not detected on carrots grown under similar conditions. The presence of *L. monocytogenes* in crop production has often been reported following described cases of listeriosis (Brandl, 2006).

*L. monocytogenes* has also been isolated from livestock (pig, sheep, goats and horses) (Nightingale et al., 2005; Nightingale et al., 2004), dairy (Fox et al., 2009) and market gardens
(Strawn et al., 2013). Intriguingly, the incidence of *L. monocytogenes* is higher in cattle farms than in small ruminant (sheep and goat) farms (Nightingale et al., 2004; Ryser and Marth, 1999). In addition, the prevalence of the pathogen varies seasonally with *L. monocytogenes* highly proliferating in this environment during the winter period (Nightingale et al., 2005). This phenomenon can be explained by the increased risk of contamination during animal housing and the use of silage during the cold season. In another study, 57 of 298 samples (cattle, milk, silage, soil and water) from 16 farms were positive for *L. monocytogenes*, registering a 19% prevalence (Fox et al., 2009). Monitoring the excretion of *L. monocytogenes* in a dairy farm showed that up to 94% of cows excreted *L. monocytogenes* at least once during the study period (Ho et al., 2007). In addition, intestinal carriage of *L. monocytogenes* and its presence in feces has been demonstrated in many animal species (Wesley, 1999). This animal carriage of *L. monocytogenes* in the agricultural environment can lead to the contamination of animal products such as raw milk (Fenlon et al., 1995; Hunt et al., 2012; Yoshida et al., 1998) or meat (Dmowska et al., 2013; Mohammed et al., 2010; Morild et al., 2011). Contamination of agricultural raw materials is a pathway for transfer of the pathogen to the food sector.

Wild animals (Gray and Killinger, 1966; Kalorey et al., 2006; Lyautey et al., 2007a), poultry, wild birds (Bouttefroy et al., 1997; Fenlon, 1985; Iida et al., 1991; Kalorey et al., 2006; Weber et al., 1995; Weis and Seeliger, 1975), dogs, cats (Iida et al., 1991; Weber et al., 1995) and rats (Iida et al., 1991) can also carry *L. monocytogenes*. The use of manure as a fertiliser can similarly increase the likelihood of isolating *L. monocytogenes* from production sites (Fenlon et al., 1996; Garrec et al., 2003; Watkins and Sleath, 1981). This is not surprising as animals are known reservoirs of the bacterium (Esteban et al., 2009; Fenlon et al., 1996; Mohammed et al., 2010).

**iii. WATER SOURCES**

*L. monocytogenes* is often detected in wastewater and effluents and the most isolated *Listeria* species from water treatment plants (Bernagozzi et al., 1994). In relation to this, studies have shown that treated sludge and effluents frequently carry *L. monocytogenes* (Garrec et al.,
The prevalence of *L. monocytogenes* in treated wastewater was estimated at 84%, of six French urban wastewater treatment plants and one composting facility, examined monthly over a 1-year period (Paillard *et al.*, 2005). During the sewage treatment process in the treatment plant, sludge is produced, which is later used in agriculture to fertilize the soil. The sludge quality used for land application is therefore a critical point for the sanitary quality of the soil. In addition, the presence of the pathogen in treated wastewater raises a possible health problem if released in the environment and/or used for crop irrigation. In contrast to rainfall, irrigation increases the chances of isolating *L. monocytogenes* from production environments (Weller *et al.*, 2015b). Reservoirs of drinking water for humans and animals, such as rivers, lakes, mountain surface water and groundwater can also carry *L. monocytogenes*, serving as a vehicle for dissemination (Bernagozzi *et al.*, 1994; Linke *et al.*, 2014; Lyautey *et al.*, 2007b; Schaffter and Parriaux, 2002; Schaffter *et al.*, 2004).

*L. monocytogenes* is often isolated from marine environments (Colburn *et al.*, 1990; Rodas-Suarez *et al.*, 2006; Rorvik *et al.*, 2000). Together with the fact that seafood has frequently been shown to carry *L. monocytogenes* (Colburn *et al.*, 1990; Gonzalez *et al.*, 2013; Johansson *et al.*, 1999; Leong *et al.*, 2015), it implies that seawater might be a source of contamination in some cases. As with rivers, it is possible that effluent and land run off may increase levels of contamination by this microorganism in coastal waters (Fenlon *et al.*, 1996; Leong *et al.*, 2015; NicAogáin and O’Byrne, 2016; Watkins and Sleath, 1981). Although *L. monocytogenes* can survive in seawater, this occurs in a temperature-dependent manner, with lower temperatures correlating with higher survival rates (Bremer *et al.*, 1998; Hansen *et al.*, 2006; Hsu *et al.*, 2005). However, other factors such as osmotic stress, predation by protozoa, nutrient availability and solar radiation should also be considered for the survival of *L. monocytogenes* in seawater (NicAogáin *et al.*, 2018; Smith *et al.*, 1994; Tedetti and Sempere, 2006).

**IV. FOOD PRODUCTS AND FOOD-PROCESSING INDUSTRY**

Contamination with *L. monocytogenes* can occur in all sectors of food-related industries,
including the dairy industry, meat processing, in raw materials as well as in the processing and distribution of finished products (Fox et al., 2011; Gelbicova and Karpiskova, 2012; Leong et al., 2017). There are different theories trying to explain the persistence of *L. monocytogenes* in food processing plants. One theory concerns the presence of particularly persistent non-dividing cells that have an increased ability to survive environmental stresses (Knudsen et al., 2013; Wen et al., 2011). Persistence could be linked to the inability to remove cells from niches within the food industry, where they can survive and grow, rather than the presence of strains with unique properties (Carpentier and Cerf, 2011). On the other hand, other studies point to the fact that bacterial persistence is more likely related to biofilm formation, since cells within biofilms are known to be more resistant to biocides and stress conditions (Holch et al., 2013; Nowak et al., 2017; Wang et al., 2015).

The ability of *L. monocytogenes* to colonize food-processing surfaces and form persistent biofilms in food industry makes this foodborne pathogen a major concern in food safety as it could serve as source of contamination. *L. monocytogenes* biofilm formation can increase its persistence for several months or even years in food industries, where it can cause recurrent cross-contamination of food products (Beresford et al., 2001; Ferreira et al., 2014; Orgaz et al., 2013; Renier et al., 2011; Tresse et al., 2007; Van Houdt and Michiels, 2010). This foodborne pathogen can attach readily to smooth surfaces in food processing environments, such as stainless steel, polystyrene and glass (Di Bonaventura et al., 2008), although it has been shown that biofilms form more readily on rougher surfaces (Silva et al., 2008). Different studies have been conducted to investigate the main sources of contamination within food processing facilities (Chen et al., 2010; Hansen et al., 2006; Ho et al., 2007; Johansson et al., 1999; Leite et al., 2006; Rivoal et al., 2010). Floors, floor drains, waste water pipes, bends in pipes, conveyor belts, rubber seals, and stainless-steel surfaces are some of the most common reservoirs of *L. monocytogenes* biofilms, as well as improperly cleaned and sanitized equipment and airborne microbiota (Di Ciccio et al., 2012; Liu et al., 2016). Operators within the facilities or different pieces of equipment may also serve as vectors for the transmission of bacteria within food processing facilities (Chen et al., 2010; Di Ciccio et al., 2012; Leite et al., 2006; Lomonaco et al., 2015).

In natural environments biofilms are typically composed of multiple bacterial species. Mixed-species biofilms with either *Flavobacterium* spp. or *Staphylococcus aureus* strains increased
the number of *L. monocytogenes* cells attaching to stainless steel compared to *L. monocytogenes* in a pure culture (Bremer *et al.*, 2001; Carpentier and Chassaing, 2004; Rieu *et al.*, 2008b). In contrast, other studies about co-cultivation of *L. monocytogenes* with food-related strains showed that some species, such as *Staphylococcus sciuri* (Leriche and Carpentier, 2000), *Pseudomonas fragi* (Norwood and Gilmour, 2001), *Enterococcus durans*, and *Lactococcus lactis* (Zhao *et al.*, 2004), were able to limit *L. monocytogenes* populations within biofilms.

Mixed-species biofilms have been found to be more resistant to disinfectants and sanitizers than mono-species biofilms. Higher resistance to the disinfectants benzalkonium chloride and peracetic acid of mixed-species biofilms of *L. monocytogenes* and *Lactobacillus plantarum* was observed, compared to the mono-species biofilms (van der Veen and Abee, 2011). However, other authors did not observe an influence of culture conditions on *L. monocytogenes* resistance to antimicrobials when it was co-cultivated with *Pseudomonas putida* (Giaouris *et al.*, 2013) or with *Salmonella enterica* (Kostaki *et al.*, 2012).

Within food production facilities, it is known that *L. monocytogenes* can survive over long periods of time. As a result of post-process contamination, *L. monocytogenes* has been isolated from a wide range of processed and cooked food (Jofré *et al.*, 2016; Vitas and García-Jalon, 2004; Vongkamjan *et al.*, 2016). The presence of the pathogen has been observed in milk storage tanks and in production equipment (Latorre *et al.*, 2010), in salmon smokers (Serio *et al.*, 2011) and in meat processing workshops (Kurpas *et al.*, 2018). Current EU regulations (EC/1411/2007) state that *L. monocytogenes* should not be detected in 25 g of food products that support its growth. Food contamination resulting from the presence of *L. monocytogenes* has a socio-economic impact for industries mostly due to the risk of causing diseases in the consumer, especially those that are immunocompromised (Cox *et al.*, 1997).

### 1.2.2. *L. MONOCYTOGENES* WITHIN THE MAMMALIAN HOST

*L. monocytogenes* is a facultative intracellular pathogen that has the capacity to transitioning from a saprophytic lifestyle to a highly virulent state within the mammalian host. Usually,
infection results from the ingestion of contaminated food and the crossing of *L. monocytogenes* through the intestinal epithelium of susceptible humans or animals. Moreover, this pathogen is also fully capable of establishing infection in the blood when inoculated intravenously, and cutaneous listeriosis infection has also been reported, albeit rarely (Godshall *et al.*, 2013; Lecuit *et al.*, 2001). It has been estimated that about 5 to 10% of the general population could be carriers of *L. monocytogenes* (Farber and Peterkin, 1991), although the incidence of infection is low in the general population despite the wide distribution of the pathogen in the environment and the relatively high frequency of isolation in foods.

### i. Listeriosis: Symptomatology and Epidemiology

Listeriosis is a life-threatening foodborne disease caused by ingestion of food contaminated with *L. monocytogenes*. Once inside the host, *L. monocytogenes* invades host cells, surviving in the gastrointestinal tract of humans and animals, multiplying intracellularly and spreading systemically by crossing intestinal, placental and blood-brain barriers (Cossart and Lebreton, 2014). The ingestion of very high numbers (10⁹ CFU) of *L. monocytogenes* by the general population might lead to an illness that is mostly expressed as gastrointestinal disorder-associated symptoms such as nausea, vomiting, diarrhoea and abdominal pain, together with mild fever, which resolve themselves within a day (Farber and Peterkin, 1991; Swaminathan and Gerner-Smidt, 2007). However, the incidence of systemic listeriosis is much higher in at-risk populations, including pregnant women and their new-borns, the elderly and immunocompromised individuals (Buchanan *et al.*, 2017). If the infection is able to develop unchecked by the immune system it can cause systemic listeriosis that presents more severe symptoms such as flu-like symptoms, abortion, meningitis or encephalitis, that lead to a high rate of hospitalizations and case fatality (approximately 20%) (Mead *et al.*, 1999; Scallan *et al.*, 2011; Vazquez-Boland *et al.*, 2001).

Over the last two decades, *L. monocytogenes* has emerged to become one of the main causes of foodborne fatalities in Europe and the USA (Goulet *et al.*, 2008; Scallan *et al.*, 2011). A considerable economic burden is caused by *L. monocytogenes* in both prevention and
treatment. In the USA, the estimated cost to the food industry to control for *L. monocytogenes* was as high as 2.4 billion dollars a year (Ivanek *et al.*, 2004), while high hospitality rates represent costs of 2.6 billion dollars annually (Cartwright *et al.*, 2013; FDA, 2018; Scallan *et al.*, 2011).

### ii. Cell Internalization

Upon ingestion of contaminated food by the mammalian host, *L. monocytogenes* reaches the intestinal epithelium where the pathogen initiates the process of entry ([Figure 1.5](#)) by binding the bacterial proteins InlA and InlB to the cellular ligands of the epithelial cells E-cadherin and Met, respectively, activating signalling cascades that lead to internalization of the pathogen (Pizarro-Cerda and Cossart, 2018). Internalin proteins (Inl) carry N-terminal leucine-rich repeat (LRR) domains containing at least three repeats of 22 amino acids, which mediate interaction between host receptor and bacterial ligands (Briere *et al.*, 2007; Gaillard *et al.*, 1991). Over 20 other internalins have been identified in *L. monocytogenes*, contributing to diverse functions including cell-to-cell spread and escape from innate immune responses (i.e., InlC) and escape from autophagy (i.e., InlK) (Dortet *et al.*, 2011; Rajabian *et al.*, 2009).

InlA displays a C-terminal LPXTG domain that supports the covalent anchor to the *L. monocytogenes* cell wall (Gaillard *et al.*, 1991), while its LRR domain interacts with the cellular receptor E-cadherin (Lecuit *et al.*, 1997; Mengaud *et al.*, 1996), a transmembrane glycoprotein present in cells of the intestine, placenta and blood-brain barrier. The interaction between InlA and E-cadherin is species-specific (Lecuit *et al.*, 1999). E-cadherin plays a key role in maintaining tissue stability, binding to other E-cadherin ligands on adjacent cells to form adherens junctions of polarized tissues. By subverting these E-cadherin homotypic interactions, *L. monocytogenes* alters host cells physiology favouring cellular invasion of the intestinal and feto-maternal barriers (Lecuit *et al.*, 2004; Lecuit *et al.*, 2001).

InlA binding promotes two successive posttranslational modifications in the cytoplasmic tail of E-cadherin, phosphorylation by the host kinase Src and subsequent ubiquitylation by the ubiquitin ligase Hakai (Bonazzi *et al.*, 2008). This promotes the adaptor Dab2 to recruit a clathrin coat, followed by the recruitment of the protein adaptor Hip1R that in turn
coordinates the recruitment of actin that together with myosin VI and unconventional myosin VIIa provide the pulling force that finally leads to bacterial internalization (Bonazzi et al., 2011; Sousa et al., 2004). Other molecules modulate actin association with E-cadherin during L. monocytogenes InLA-dependent invasion such as β- and α-catenins (Lecuit et al., 2000; Sousa et al., 2005), cortactin and Src (Sousa et al., 2007), and the PI 3-kinase, which is constitutively active in the intestinal barrier, in contrast to in the placenta where InlB is required for PI 3-kinase activation and InLA-mediated cell invasion (Disson et al., 2008; Gessain et al., 2015).

Lastly, the interaction between InlA and E-cadherin induces a rearrangement of the actin cytoskeleton of the host cell is induced (Bonazzi et al., 2008; Camejo et al., 2011; Lecuit et al., 2000). The length of the InlA protein produced influences determines the success of infection establishment as full length functional InlA is produced by virulent 4b and 1/2b isolates, whereas the rarity of the 1/2c strains among clinical strains may be related to a truncated internalin (Jacquet et al., 2004).

The second L. monocytogenes invasion protein InlB binds to Met, a receptor for HGF (hepatocyte growth factor) allowing the entry of the pathogen into nonpolarized epithelial cells (Dramsi et al., 1995; Gaillard et al., 1991), and collaborating with InlA during placental invasion (Disson et al., 2008; Gessain et al., 2015). The InlB C-terminal region is characterized by the presence of glycine-tryptophan (GW) repeats that favour loose binding to bacterial membrane lipoteichoic acids (Braun et al., 1997; Jonquieres et al., 1999) and peptidoglycan-bound teichoic acids (Carvalho et al., 2018). The N-terminal region of InlB displays LRRs that are essential for binding to the hepatocyte growth factor receptor Met in a species-specific manner (Khelef et al., 2006; Shen et al., 2000). Met is a tyrosine kinase receptor, and through interaction with InlB it autophosphorylates itself, and recruits a number of protein adaptors (Gab1, Shc, Cbl, and CrkII) that in turn activate the phosphatidylinositol (PI) 3-kinase and cell invasion by L. monocytogenes in an InlB-dependent manner (Basar et al., 2005; Ireton et al., 1999; Jiwani et al., 2012; Sun et al., 2005). At its final stage, the interaction between InlB and Met triggers a signalling cascade that favours the rearrangement of the actin cytoskeleton in a Arp2/3 complex dependent manner (Bierne et al., 2001; Ireton et al., 1996; Ireton et al., 1999).

Both these processes lead to bacterial engulfment and internalization in a membrane-bound vacuole into the cytoplasm of host phagocytic and non-phagocytic cells (Figure 1.5). Overall,
InlA is required for entry in non-phagocytic cells, specially goblet cells of the intestinal lining (Lecuit et al., 2001), while the presence of InlB contributes to invasion of other non-phagocytic cells as trophoblasts or of the Peyer’s patches, though it is not critical for crossing the intestinal epithelium (Pentecost et al., 2010). Besides internalins, numerous other proteins are also involved in host cell adhesion and/or invasion processes such as the autolysins Ami, Auto and IspC (Cabanes et al., 2004; Milohanic et al., 2001; Wang and Lin, 2008), the lipoteichoic acid modifiers GtcA and DltA (Abachin et al., 2002; Promadej et al., 1999), the lipoprotein LpeA (Reglier-Poupet et al., 2003), the lipoprotein transferase Lgt (Machata et al., 2008), the lysylphosphotidylglycerol modifier MprF (Thedieck et al., 2006), and the surface adhesins or invasins Vip, Lap, LapB, and FbpA (Dramsi et al., 2004; Jagadeesan et al., 2011; Martins et al., 2012; Reis et al., 2010) contribute to adherence and invasion.

Listeriolyisin O (LLO) is sufficient to induce L. monocytogenes internalization via the formation of pores in the cell membrane and perturbation of host cell tyrosine kinase signalling, F-actin polymerization and dynamin (Vadia et al., 2011). Through the secretion of LLO (Figure 1.5), extracellular bacteria can trigger physiological modifications of the host cells by forming pores in the cell membranes that induce a transient influx of extracellular calcium within host cells, which in turn correlates with increased cell invasion (Dramsi and Cossart, 2003). Membrane perforation by LLO facilitates L. monocytogenes translocation to the cytoplasm, and it controls the vacuolar pH and ions concentration, delaying the maturation of the bacteria-containing compartment and inhibiting lysosomal fusion (Henry et al., 2006; Shaughnessy et al., 2006).
Figure 1.5. *L. monocytogenes* intracellular stages of infection. *L. monocytogenes* enters non-phagocytic cells through receptor mediated endocytosis. Interaction of internalins InIA and InIB with host cells receptors favours actin recruitment, remodeling of the plasma membrane, and bacterial engulfment. The secreted pore-forming toxin LLO and the surface molecule ActA are also implicated in bacterial endocytosis. Internalized *L. monocytogenes* is contained in a vacuole and through transcytosis is translocated to the lamina propria. Several virulence factors, including the pore-forming LLO, the metalloprotease Mpi, the phospholipases PlcA and PlcB, and the pheromone pPplA, promote disruption of the vacuole and *L. monocytogenes* release in the cytosol. Once in the cell cytosol, bacteria take advantage of host metabolites, including transport of glucose-1-phosphate via the transporter Hpt. The surface protein ActA promotes actin-based motility and the secreted protein InIC favours reduction of plasma membrane cortical tension, allowing invasion of neighbouring cells. LLO, PlcA and PlcB contribute to the disruption of the double-membrane vacuole. Extracellular LLO is able to modulate different cellular functions, including mitochondrial fission, lysosomal permeabilization, protein SUMOylation, ER stress, DNA damage, calcium influx and chromatin remodelling. PlcA, PlcB and ActA, have been implicated in the resistance to autophagy. Adapted from Pizarro-Cerda et al., 2018.
iii. INTRACELLULAR MULTIPLICATION AND DISSEMINATION

Several bacterial virulence factors (Figure 1.5), including the phospholipases PlcA and PlcB, the pore-forming toxin LLO, the actin-polymerizing surface protein ActA, and the internalin InlC compromise various host cellular functions thereby contributing to different stages of L. monocytogenes cell-to-cell spread. Once internalized, L. monocytogenes secretes LLO and two Mpl-activated phospholipases C PlcA and PlcB to perforate the membrane-bound vacuole and translocates to the host cell cytoplasm (Figure 1.5) (Dramsi and Cossart, 2003; Geoffroy et al., 1991; Mengaud et al., 1991a; Mengaud et al., 1991b). The pheromone pPplA is also secreted by L. monocytogenes and triggers the production of an unknown factor that accelerates vacuolar disruption (Figure 1.5) (Xayarath et al., 2015). Reversible lysogeny has also been proposed to modulate bacterial gene expression. Indeed, excision of the prophage A118 from the genome of L. monocytogenes EGD-e inside the internalisation vacuole results in the recovery of full-length comK and expression of the competence machinery that promotes bacterial vacuolar escape (Rabinovich et al., 2012). On the other hand, L. monocytogenes in the intestinal epithelium (e.g. goblet cells) cannot escape from this compartment and is directly transcytosed to the lamina propria, where the bacteria disseminate systemically (Nikitas et al., 2011). However, L. monocytogenes residency and persistence in vacuolar compartments has also been described (Bierne et al., 2018; Kortebi et al., 2017). In response to the changes it faces once in the host cell cytoplasm, L. monocytogenes adapts its metabolism to the nutrients and metabolites available in its surroundings and its surface goes through some changes leading to increased resistance to cell autodefense mechanisms. L. monocytogenes uses glucose-1-phosphate, the primary degradation product of glycogen widely available in mammalian cells (Ripio et al., 1997). The hexose phosphate transporter Hpt is responsible for the uptake of glucose-6-phosphate in the cytoplasm of host cells, playing a key role in L. monocytogenes virulence (Figure 1.5) (Chico-Calero et al., 2002).

Once out of the vacuole, cytoplasmic L. monocytogenes firstly multiplies and then it activates a flagellum-independent motility mechanism through the polymerization of actin by the actin-polymerizing surface protein ActA. This cytoplasmic movement favours the avoidance of autophagosomes, as the assembling of a protective barrier of polymerized actin or ActA sequestered Arp2/3 helps to prevent accumulation of autophagy signalling molecules (Figure
1.5) (Birmingham et al., 2007; Perrin et al., 2004; Yoshikawa et al., 2009). Actin-based motility helps to propel the bacterium through the cytoplasm until it eventually propels itself into the host cell membrane and forms a protrusion into a neighbouring cell by pushing through the membrane, ultimately leading to bacterial entrapment in a double-membrane vacuole that is then disrupted (Tilney and Portnoy, 1989). Internalin C favours bacterial protrusion formation by inhibiting the recruitment of actin regulatory proteins and therefore relieving cortical membrane tension formation (Gianfelice et al., 2015; Polle et al., 2014; Rajabian et al., 2009). Once more, L. monocytogenes escapes the bacterial containing vacuole using LLO, PlcA and PlcB, and begins to replicate again (Figure 1.5) (Alberti-Segui et al., 2007).

The cell-to-cell spreading mechanism allows L. monocytogenes to disseminate throughout contaminated tissues while avoiding exposure to humoral immunity (Cossart and Sansonetti, 2004; Kuehl et al., 2015). Macrophages allow bacterial evasion of the humoral immune response system as well, and LLO and ActA are essential to this process, playing a prominent role in escape from the macrophage phagosome (Disson and Lecuit, 2013; Hamon et al., 2007). The phospholipase PlcB also facilitates efficient bacterial internalization in macrophages by inducing calcium influx (Wadsworth and Goldfine, 1999). Calcium influx leads to mitochondrial fragmentation and to the modulation the bioenergetic state of host cells, which triggers L. monocytogenes cell invasion (Stavru et al., 2011). Prior to its entry into host cells (Figure 1.5), L. monocytogenes modulates the ER condition in a LLO-dependent manner by activation of the unfolded protein response, a signalling cascade that maintains the function of ER under stress (Pillich et al., 2012). The integrity of lysosomes is also compromised by extracellular LLO, which induces permeabilization and release of lysosome content in the cytoplasm, although its impact on infection remains to be determined (Malet et al., 2017).

Essential posttranslational modifications of host cell proteins are also targeted by L. monocytogenes through LLO activity improving bacterial infection. Following calcium influx prompted by extracellular LLO, cellular protein SUMOylation is downregulated through the proteasome-independent degradation of the E2 enzyme Ubc9 together with the proteasome-dependent degradation of some sumoylated proteins (Figure 1.5) (Ribet et al., 2010). DNA stability of host cells is also modulated by L. monocytogenes in a LLO-dependent manner through the induction of DNA strand breaks and degradation of the DNA damage sensor
Mre11, promoting a delay in the cell cycle that favours bacterial intracellular replication (Figure 1.5) (Leitao et al., 2014; Samba-Louaka et al., 2014). Similarly, proteasomal degradation of the human telomerase reverse transcriptase is also triggered by extracellular LLO-induced calcium influx (Samba-Louaka et al., 2012).

L. monocytogenes undertakes several mechanisms to fine-tune host gene expression: (i) control of host transcription through bacterial nucleomodulins LntA (Lebret on et al., 2011) and OrfX (Bierne et al., 2009; Prokop et al., 2017); (ii) inhibition of NF-κB translocation to the nucleus (Gouin et al., 2010), (iii) LLO-induced dephosphorylation of histone H3, deacetylation of histone H4 (Hamon et al., 2007) and functional modulation of the promyelocytic leukemia protein nuclear bodies (Ribet et al., 2017), and (iv) SIRT2-dependent deacetylation of histone H3 through an InlB/PI 3-kinase pathway (Eskandarian et al., 2013). Using all the different strategies mentioned above, L. monocytogenes is able to takeover multiple host cellular functions including receptor signalling, membrane trafficking, cytoskeletal rearrangements, organelle dynamics, DNA stability, and gene transcription, favouring invasion and intracellular growth. Overall, the work that has been undertaken to provide important insights into infection biology, immunity and host cell biology over the years, made of L. monocytogenes a model organism for the study of pathogenesis (Cossart and Archambaud, 2009).
1.3. **COMMUNICATION AND AUTO-INDUCTION IN *L. MONOCYTOGENES***

Over time, bacteria have evolved mechanisms to sense the surrounding environment, integrate these signals and induce regulation of gene expression to thrive under ever-changing conditions. The discovery of cell-to-cell communication within bacterial populations has changed the way we understand microbial populations functioning. The first description of a self-induced bacterial communication system postulated that an extracellular molecule contributed to physiological changes in a bacterial population (Nealson *et al.*, 1970; Tomasz, 1965). Cellular communication is based on the synthesis, diffusion and perception of signaling molecules in the extracellular medium that lead to transcriptional regulation of target genes. A signaling molecule is a metabolite that is produced during a specific stage of bacterial growth, under certain physiological conditions, or in response to an environmental change, accumulating in the extracellular environment where it is detected by a specific receptor triggering a cellular response that induces physiological changes (Winzer *et al.*, 2002). In Gram positive bacteria, signal molecules are usually short peptides processed by transmembrane proteins or other proteases (Dunny and Leonard, 1997). Once secreted, these autoinducers accumulate and interact with a two-component system; after binding to the sensor histidine kinase, the signal is translocated from the outside to the cytoplasm via a phosphorylation cascade and the phosphorylated regulator affects transcription of target genes (Surette and Bassler, 1999).

Three different communication theories have been proposed, (i) Quorum sensing (QS), the phenomenon whereby bacteria sense the accumulation of signalling molecules in the surrounding environment and respond to cell population density by coordinating gene expression to make a synchronised response (Fuqua *et al.*, 1994; West *et al.*, 2007); (ii) Diffusion sensing (DS), which suggests that cells to assess when producing exofactors will be directly beneficial to itself, in response to the rate of autoinducers diffusion in the environment (Redfield, 2002); and (iii) Efficiency sensing (ES), that explains autoinduction and signalling in complex environments thus unifying population density (QS) and spatial confinement (DS) in a global theory of cell-cell communication (Hense *et al.*, 2007). Two communication systems have been extensively studied in Gram-positive bacteria, the
development of competence associated with the autoinducer ComX identified in *Bacillus subtilis* (Magnuson et al., 1994) and the accessory gene regulator (agr) system first described in *Staphylococcus aureus* (Recsei et al., 1986). *In silico* analysis indicated orthologues of merely some competence genes of *B. subtilis* in *L. monocytogenes* EGD-e, however the full agr operon from *S. aureus* was identified in *L. monocytogenes* genome (Autret et al., 2003).

1.3.1. **The Agr System and Transcriptional Regulation**

In order to adapt to environmental fluctuating conditions, *L. monocytogenes* possesses 15 complete two-component systems and a number of regulatory circuits (Guariglia-Oropeza et al., 2014; Williams et al., 2005). One of these systems is encoded by the agr locus, a bacterial communication system consisting of a quorum sensing module paired with a classical two-component system that has been shown to be involved in virulence, biofilm formation, and survival in the outdoor environment (Autret et al., 2003; Riedel et al., 2009; Rieu et al., 2007; Vivant et al., 2015).

The Agr system of *S. aureus* is a peptide exchange-based communication system, whose genes are organized into two divergent transcription units, RNAII and RNAIII, placed under the control of two promoters, P2 and P3, respectively. The RNAII transcription unit is an operon composed of the four agrBDCA genes that encode the Agr communication system. The agrD gene encodes the precursor propeptide of the self-inducing peptide (AIP) that is processed and exported by agrB-encoded transmembrane protein in association with the Sps peptidase. Together, AgrC and AgrA constitute a classical two-component system for which AgrC is the transmembrane receptor histidine kinase and AgrA the transcriptional response regulator (Lina et al., 1998). At high cell density, the detection of extracellular AIPs by the AgrC receptor leads to phosphorylation of the latter and transfer of the phosphate group to the AgrA transcriptional regulator. Its phosphorylation allows the activation of P2 and P3 promoters by binding phosphorylated AgrA on repeated sequences located in the intergenic region that separates the two promoters (Reynolds and Wigneshweraraj, 2011). The agrBDCA operon
thus ensures its self-induction and the transcription unit RNAIII produces a regulatory RNA which is the effector of the Agr operon.

Figure 1.6. Diagram of the Agr system of L. monocytogenes. AgrB and Sps process AgrD into a secreted cyclic autoinducing peptide (AIP), which is later detected by the sensor kinase AgrC that in turn induces a phosphorylation cascade resulting in the activation of the regulator AgrA. Adapted from Garmyn et al., 2009.

The Agr system is not common to all Firmicutes, however differences exist between the Agr system of S. aureus and its orthologous systems in L. monocytogenes such as the absence of RNAIII in the genome (Autret et al., 2003; Wuster and Babu, 2008). With the exception of L. grayi, the four agrBDCA genes are present in the Listeria genus with a high level of conservation (Nakayama et al., 2009). The organization of the four genes is also similar to the S. aureus operon (Figure 1.6), where agrB encodes a transmembrane protein allowing the AgrD propeptide to mature to a cyclic autoinducing peptide (AIP), whereas the detection of extracellular AIP by AgrC histidine kinase induces transcriptional regulation by activation of the AgrA regulator (Autret et al., 2003; Zetzmann et al., 2016).
The structure of AIPs is available for a limited number of species, including \textit{S. aureus}, \textit{E. faecalis}, \textit{L. plantarum} and \textit{Clostridium acetobutylicum} (Nakayama \textit{et al.}, 2009; Novick and Geisinger, 2008; Steiner \textit{et al.}, 2012; Sturme \textit{et al.}, 2005). Recently, the structure of the native AIP of the \textit{L. monocytogenes} EGD-e, encoded by \textit{agrD}, was firstly identified as consisting of a five-membered thiolactone ring, which has autoinducing activity (Zetzmann \textit{et al.}, 2016). The structural diversity of AIPs within the species \textit{S. aureus} (four specificity groups of strains with different AIPs) exhibited cross-inhibition (Novick and Geisinger, 2008). However, the AgrD propeptides of the genus \textit{Listeria} are conserved, with the species \textit{L. monocytogenes}, \textit{L. innocua}, \textit{L. ivanovii}, \textit{L. welshimeri}, \textit{L. seeligeri}, and \textit{L. marthii} have identical predicted AIP sequences, which suggests cross-reactivity (Zetzmann \textit{et al.}, 2016).

\subsection*{1.3.2. Importance of the Agr System in \textit{L. monocytogenes} Adaptation}

A very limited number of studies using differential transcriptomic approaches have been attempting to define the \textit{agr} regulon and to determine its importance in the physiology of \textit{L. monocytogenes}. These studies compared the expression profiles of the \textit{agrD} (Riedel \textit{et al.}, 2009) or the \textit{agrA} (Garmyn \textit{et al.}, 2012) gene knockout mutants to those of the wild-type strain \textit{L. monocytogenes} EGD-e. Deletion of \textit{agrD} resulted in a global change in gene expression. During the exponential phase at 37 °C, transcription of 121 genes was significantly different in the \textit{ΔagrD} mutant, as many of these genes encoded regulatory proteins (n= 58) (Riedel \textit{et al.}, 2009). Similar results were observed in the \textit{ΔagrA} mutant grown under the same conditions (Garmyn \textit{et al.}, 2012), as a total of 712 genes were found to be differently expressed in this mutant, mostly encoding proteins involved in regulatory mechanisms and virulence (Garmyn \textit{et al.}, 2012). Altogether, these results suggested that the \textit{agr} system plays a global role in the regulation of \textit{L. monocytogenes} gene expression.

The comparison of transcriptomes at different temperatures (37°C and 25°C) made it possible to evaluate the impact of AgrA in temperature adaptation (Garmyn \textit{et al.}, 2012). Inactivation of \textit{agrA} led to significant transcriptome rearrangement at 37°C, which were not so evident at 25°C (Garmyn \textit{et al.}, 2012). Overall, these studies suggest that the Agr system is involved in
the regulation of biological functions allowing \textit{L. monocytogenes} to adapt to the various environments it encounters.

\textbf{i. Virulence}

A few studies have been attempting to understand the roles of the Agr system in the biology of \textit{L. monocytogenes}, providing information sometimes contradictory, about the connection between the Agr system and virulence (Autret \textit{et al.}, 2003; Riedel \textit{et al.}, 2009; Williams \textit{et al.}, 2005). The deletion of \textit{agrA} did not influence the production of virulence factors ActA and Pc-PLC, or \textit{L. monocytogenes’ in vitro} invasion ability; however, a slight reduction in the secretion of the LLO was observed solely in exponentially grown cells (Autret \textit{et al.}, 2003; Williams \textit{et al.}, 2005), suggesting that the regulation of the extracellular proteins expression by Agr occurs only during the exponential phase of growth. Similarly, \textit{in vitro} intracellular penetration and multiplication in epithelial cells (Caco-2) and hepatocytes (HepG-2) were not changed in the \textit{ΔagrA} mutant (Autret \textit{et al.}, 2003). On the other hand, the deletion of \textit{agrD} resulted in an altered virulence phenotype, decreasing the adhesion and invasion capacity of Caco-2 epithelial cells for the \textit{ΔagrD} mutant, which correlated with the minor amount of InlA protein detected in the cell wall of this mutant strain (Riedel \textit{et al.}, 2009). In addition, the decrease in the expression of the PrfA regulator shown in the \textit{ΔagrD} mutant together with a reduced \textit{in vivo} infection suggested that there is a defect in the transcriptional activation of virulence genes in the \textit{ΔagrD} mutant (Riedel \textit{et al.}, 2009). Overall, these studies suggest that the Agr system induces physiological changes in \textit{L. monocytogenes} that impact virulence.

\textbf{ii. Biofilm formation}

The potential role of the Agr system in the adhesion of \textit{L. monocytogenes} to surfaces and in biofilm formation has also been scrutinized (Kumar \textit{et al.}, 2009; Riedel \textit{et al.}, 2009; Rieu \textit{et al.}, 2007). The characterization of deletion mutants \textit{ΔagrA} and \textit{ΔagrD} has demonstrated that this two-component system is important at the initial stages of biofilm formation, when the \textit{agr} system is activated, impairing the adhesion of the cells of the mutants that is reflected in a
significant decrease in the number of cells adhering to the glass surface relative to the wild-type strain (Rieu et al., 2008b; Rieu et al., 2007). Similarly, biofilm formation on polystyrene is affected for ΔagrA and ΔagrD mutants during the first 24 hours of incubation (Riedel et al., 2009; Rieu et al., 2007). Studies have consistently showed that these mutations did not affect mature biofilm formation, still a single study showed a defect in mature biofilm development for the ΔagrA mutant (Kumar et al., 2009). It is difficult to compare these studies because of differences in experimental conditions (medium, temperature, and incubation time); nevertheless, the mutations in the agr system consistently limited adhesion and biofilm formation of L. monocytogenes in different media and temperatures, excepting at 37°C when the phenotype was reversed. The amount of biofilm was greater when the agr system was not functional, indicating that the deletion of the agrA causes a major regulation defect that results in a physiological upheaval (Garmyn et al., 2012).

A heterogeneous activation of the Agr system during the growth of L. monocytogenes EGD-e has been demonstrated in situ, in both static and fluidic conditions, using a Pagr-gfp reporter system. In static conditions, only 1% of cells in a 48 hours biofilm expressed GFP whereas in dynamic conditions, up to 80% of cells expressed it, and mostly localized in the superficial regions of the biofilm where the concentration of AIP should not be maximal (Rieu et al., 2008a). Likewise, in mid-exponential phase, a total of 15% of the cells expressed GFP, while at the end of the exponential growth phase the percentage of fluorescent cells reaches 28% of the population (Garmyn et al., 2011). While growing at 37°C the percentage of gfp-expressing cells increased to 49% of the population. In addition, nutrient availability was also shown to influence Agr activation, with the percentage of fluorescent cells increasing in nutrient-poor media but decreased in rich media (Garmyn et al., 2011). A bet-hedging theory has been suggested for the Agr system activation in L. monocytogenes (Garmyn et al., 2011), which attempts to explain the generation of offspring with different phenotypes as a mechanism that facilitates survival of clonal populations to ever-changing environmental conditions (Veening et al., 2008a; Veening et al., 2008b).
iii. SAPROPHYTIC LIFESTYLE

Not much is known about the specific role of the agr system in *L. monocytogenes* saprophytic lifestyle. Limited nutrients availability in the telluric environment prompted *L. monocytogenes* to develop mechanisms for using a large range of carbon and nitrogen sources widely present in nature (e.g., cellulose and its hydrolysis by-products, and chitin polymers) to synthesize specific enzymes required for its catabolism. The chitinolytic system of *L. monocytogenes* allows the degradation chitin, the second most abundant carbohydrate in nature, and use it as a nutrient source (Beier and Bertilsson, 2013; Gooday, 1990a; Gooday, 1990b). This chitinolytic system comprises two chitinases (ChiA and ChiB) and a putative lytic polysaccharide monooxygenases (Lmo2467), which have an additional role in virulence by promoting infection (Frederiksen et al., 2013; Leisner et al., 2009; Leisner et al., 2008). A role for the Agr system in the regulation of the chitinolytic activity of *L. monocytogenes* was identified. The implication of the Agr system on efficient chitin hydrolysis regulation was postulated as the deletion of agrD dramatically decreased chitinolytic activity on agar plates (Paspaliari et al., 2014). During stationary phase of growth, Agr was specifically induced in response to chitin addition to the extracellular medium, as agrD was found to regulate the number of chiA transcripts (Paspaliari et al., 2014). However, despite the fact that the transcript levels of chiB did not depend on agrD, the extracellular protein levels of both chitinases were found reduced in the ΔagrD mutant. A potential regulatory effect of Agr on chiA mediated through the agr-repressed small RNA (sRNA) LhrA was also suggested, since this sRNA is known to repress chiA translation (Paspaliari et al., 2014).

Additionally, the Agr communication system of *L. monocytogenes* was shown to be required for optimal adaptation and survival in soil. Despite the agr system inhibition, soil’s natural microbiota further impacts the survival of *L. monocytogenes* in soil, as either the deletion of agrA or agrD, did not affect population dynamics in sterilized soil, although survival was altered in biotic soil (Vivant et al., 2014). This suggested that the Agr system was implied in the fitness of *L. monocytogenes* in the complex soil biotic environment, which was supported by transcriptomic analysis, as adaptation to the soil environment required an extensive re-profiling of gene expression and genes coding proteins involved in cellular processes and intermediary metabolism including chitinases and β-glucosidases were found upregulated
(Piveteau et al., 2011). Co-incubation experiments showed that the ability to respond to Agr communication was beneficial for listerial cells to compete, allowing to further conclude that in soil, the Agr system controls private goods (intracellular factors) rather than public goods (exo-products) (Vivant et al., 2014).

$L.\ monocytogenes\ \DeltaagrA$ mutant displayed significantly reduced survival than the wild-type during adaptation in a set of ten biotic soils microcosms (Vivant et al., 2015). Moreover, differential transcriptome analyses showed large alterations of the transcriptome when AgrA was not functional during adaptation to biotic soil environments, 578 protein-coding genes (involved in cell envelope and cellular processes), and an extensive repertoire of sRNAs were differentially transcribed (Vivant et al., 2015). Comparatively, transcriptomic differences under sterilized soil conditions were highly reduced (genes and 29 sRNAs), suggesting that the response regulator AgrA exerts important roles during the saprophytic life of $L.\ monocytogenes$ through re-shaping gene expression at the transcriptional level (Vivant et al., 2015).
1.4. σ^B- DEPENDENT GENERAL STRESS RESPONSE OF L. MONOCYTOGENES

Bacteria enclose different mechanisms for overcoming the diverse stresses encounters in the environment. The RNA polymerase sigma factors tightly regulate gene transcription determining the genes that are transcribed at any time by directing the transcriptional machinery to the appropriate promoter sequences. Of the five sigma factors present in most L. monocytogenes strains, σ^A is the principal housekeeping sigma factor, while σ^B, σ^C, σ^H, and σ^L alternative sigma factors are utilized under specific environmental stresses (Glaser et al., 2001; O'Byrne and Karatzas, 2008). The σ^B factor controls the general stress response in L. monocytogenes and it has the largest regulon of the four alternative sigma factors, controlling the transcription of almost three hundred genes (approximately 10% of the genome) (Chaturongakul et al., 2011).

The sigB locus of L. monocytogenes has shown high levels of sequence homology with B. subtilis genomes (Ferreira et al., 2014; Wiedmann et al., 1997). It has been known that B. subtilis senses stress through a complex called the stressosome (Marles-Wright et al., 2008; Marles-Wright and Lewis, 2010); evidence for the existence of a stressosome complex within L. monocytogenes comprised of RsbR (Regulator of Sigma B) and its paralogs, along with RsbS and RsbT, has only recently been obtained (Impens et al., 2017). 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. Recently, the structure of the stressosome as well as its mechanism of activation in L. monocytogenes was described for the first time (Williams et al., 2019).

With time, numerous studies contributed on defining the σ^B regulon (Abram et al., 2008a; Abram et al., 2008b; Kazmierczak et al., 2003; Raengpradub et al., 2008; Toledo-Arana et al., 2009; Wemekamp-Kamphuis et al., 2004; Wurtzel et al., 2012). Collectively known as the General Stress Response (GSR) regulon, the genes under the control of σ^B are well defined and many contribute to a variety of stress resistance mechanisms including acid tolerance (Wemekamp-Kamphuis et al., 2004; Wiedmann et al., 1997), bile tolerance (Begley et al., 2006; Zhang et al., 2011), cell wall acting antimicrobials (Begley et al., 2006), gastrointestinal
tract (Toledo-Arana et al., 2009), osmoregulation (Fraser et al., 2003; Sue et al., 2004), visible light (O'Donoghue et al., 2016; Ondrusch and Kreft, 2011; Tiensuu et al., 2013) and virulence (Kazmierczak et al., 2006; Kim et al., 2005; Kim et al., 2004; Rauch et al., 2005). Despite being required for resistance to numerous stresses, the continual activation of $\sigma^B$ was shown to be deleterious to the cell (Min Kang et al., 1996). Competition between the housekeeping sigma factor, $\sigma^A$, and the alternative sigma factor $\sigma^B$ for RNA polymerase has been suggested, as removal of this competition lead to more cell resources available and to consequential cell growth (O'Byrne and Karatzas, 2008).

### 1.4.1. The Stressosome and the $\sigma^B$ Signaling Cascade

The stressosome of *B. subtilis* is a protein complex (1.8 megadalton) that acts as a signal integration hub, enabling the activation of the $\sigma^B$ signalling cascade in response to environmental stress (Marles-Wright et al., 2008). On a total, each stressosome comprises approximately 40 copies of RsbR and 20 copies each of RsbS and RsbT (Marles-Wright and Lewis, 2010; Pane-Farre et al., 2017). Composed of a core region made up of RsbS:RsbT complexes, surrounded by RsbR protein units, 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 (Figure 1.7) (Chen et al., 2003; Marles-Wright and Lewis, 2010). In addition to RsbR, four other proteins (RsbRA, RsbRB, RsbRC, RsbRD and YtvA) with high levels of sequence similarity to RsbR are thought to co-exist with RsbR in the stressosome complex of *B. subtilis*; all of which have homologs in *L. monocytogenes* designated RsbR (*lmo0889*), Lmo0161, Lmo0799, Lmo1642 and Lmo1842 (Ondrusch et al., 2011).

In an unstressed cell, the anti-anti-sigma factor RsbV is phosphorylated and unable to bind to the anti-sigma factor RsbW that in turn sequesters $\sigma^B$ inhibiting its interaction with RNA polymerase (Ferreira et al., 2001; Yang et al., 1996). However, in the presence of a stressful event, RsbT dissociates from the RsbS:RsbR complex, binding to RsbU instead, which in turn acts as a phosphatase, dephosphorylating RsbV that is then binds to RsbW, leaving $\sigma^B$ free to
bind to the core enzyme of RNA polymerase, and finally allowing transcription of the $\sigma^B$ regulon (Hecker et al., 2007; Marles-Wright et al., 2008; Yang et al., 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 et al., 2003; Liebal et al., 2013).

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, by complexing with the N-terminal region of RsbU following its dissociation from the stressosome (Delumeau et al., 2004), it has been shown that RsbU, but not RsbT, has an essential role in responding to energy stress via activation of $\sigma^B$ (Shin et al., 2010). Moreover, genetic evidences suggested that RsbT is essential for the activation of $\sigma^B$ (Chaturongakul and Boor, 2004). In *L. monocytogenes*, RsbV was shown to be required for the activation of $\sigma^B$ in response to several environmental stresses such as synthetic gastric fluid, acid (pH 2.5) and cumene hydrogen peroxide (Chaturongakul and Boor, 2004), as osmotic and ethanol stress (Chaturongakul and Boor, 2006), while improving growth rate under mild osmotic, acid (pH 4.5), and alcohol stress (Zhang et al., 2013). In the absence of RsbV, RsbW must likely remain bound to $\sigma^B$ that is therefore unable to interact with the transcriptional machinery and initiate transcription of the GSR genes required to induce a protective response.

The existence of a stressosome complex in *L. monocytogenes* has been recently confirmed, together with a newly identified miniprotein, Prli42, that anchors RsbR to the bacterial membrane by interacting with the N-terminal domain of RsbR, and is essential for $\sigma^B$ activation in response to oxidative stress (Impens et al., 2017). Although there is a high level of variability in the N-terminal structures of RsbR and its paralogues (Murray et al., 2005), a level of redundancy in their sensing function has been shown, as all of the paralogues are able to sense and contribute individually to ethanol stress response (Cabeen et al., 2017; Kim et al., 2004).
Figure 1.7. Schematics of the sigB operon and model of the $\sigma^B$ regulatory mechanism. (A) Each gene in the sigB operon of L. monocytogenes is represented by an open arrowhead. Transcription can be initiated from either of the two promoters (angled arrowheads), and a putative terminator sequence has been identified (stem-loop structure). (B) Upon a stressful signal, RsbR and RsbS are phosphorylated by the kinase RsbT, causing RsbT dissociation from the stressosome complex. RsbT then binds to RsbU that becomes active as a phosphatase thereby facilitating the dephosphorylation of the anti-anti sigma factor RsbV. The anti-sigma factor, RsbW, has a higher affinity for RsbV in its unphosphorylated state, leading to its dissociation from $\sigma^B$. As so, $\sigma^B$ is then free to bind to the RNA polymerase, and initiate transcription of the $\sigma^B$ gene regulon in a stressed cell. Extracted from Dorey et al., 2019 with permission.
Using cryo-electron microscopy, Williams and colleagues (2019) have recently determined the molecular structure of the stressosome of *L. monocytogenes*. According to this model, the stressosome consists of multiple copies of three proteins RsbR, RsbS and RsbT, which are anchored to the membrane by Prli42. A key phosphorylation site on RsbR is partially hidden by an RsbR flexible loop, whose “open” or “closed” position can modulate stressosome activity. Upon a stressful signal is transduced to the stressosome via Prli42, initiates some phosphorilation modifications that end up with RsbT being released from the stressosome complex and binding to RsbU that becomes active as a phosphatase, and dephosphorylates RsbV. The anti-sigma factor, RsbW, has a higher affinity for RsbV in its unphosphorylated state, leading to its dissociation from σB. Consequently, σB is then free to bind to the RNA polymerase, and initiate transcription of the σB gene regulon in a stressed cell (Williams *et al.*, 2019).

### 1.4.2. ROLE OF σB IN *L. MONOCYTOGENES* UNDER DIFFERENT STRESSES

Several studies have been attributing a role to σB in coordinating the response to different stresses in *L. monocytogenes* such as osmotic (Becker *et al.*, 1998; Fraser *et al.*, 2003; Utratna *et al.*, 2011), pH (Wemekamp-Kamphuis *et al.*, 2004; Zhang *et al.*, 2011), temperature (Liu *et al.*, 2002), light (O’Donoghue *et al.*, 2016; Tiensuu *et al.*, 2013) and oxidative stress (Ferreira *et al.*, 2001). In addition to its role in GSR, σB has been also implicated in virulence (Nadon *et al.*, 2002; Toledo-Arana *et al.*, 2009), saprophytism (Gorski *et al.*, 2011), and in biofilm formation.

#### i. ACID STRESS

In both saprophytic and pathogenic lifestyles, *L. monocytogenes* often encounters a wide range of pH values. Within the human host, the bacterium is subjected to the highly acidic environment of the stomach (pH ~2), and also less acidic environment of the duodenum (pH ~6). *L. monocytogenes* possesses a wide array of systems it can employ in order to overcome acid stress, including the adaptive acid tolerance response (ATR) (Davis *et al.*, 1996; O’Driscoll
et al. 1996), the glutamate decarboxylase (GAD) system (Cotter et al., 2001), and the arginine deaminase system (Ryan et al., 2009), all of which at least partially σ^B-dependent. The extent to which σ^B is required is dependent upon the level of stress encountered, and the protective mechanism required for the response.

The ATR mechanism of L. monocytogenes allows adaptation and survival to lethal acidic conditions after de novo protein synthesis during a previous exposure to mild acidic conditions (Davis et al., 1996). The absence of pre-exposure to mild acid conditions or the deletion of sigB resulted in a dramatic reduction in cell numbers over 3h exposure to pH 2.5, compared to the wild-type (Ferreira et al., 2001). After exposure to pH 4.5 for 1h prior to exposure to pH 2.5, the wild-type and ΔsigB mutant cells showed 10-fold and 100-fold reductions, respectively, after 3h (Ferreira et al., 2001). Moreover, a greater requirement for σ^B was noticed as the cells approached stationary phase compared to exponential phase (Ferreira et al., 2003).

In L. monocytogenes, transcription of GAD system encoding genes (gadA-E), except for gadA, is induced after exposure to acidic conditions, and two functional σ^B-promoters upstream from the gadCB operon and gadD were identified (Wemekamp-Kamphuis et al., 2004). Upon exposure to acid, the 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 three Gad enzymes, GadD1-D3 (Gahan and Hill, 2014). This last decarboxylation reaction consumes a proton, contributing to the reduction of the acidity of the cell cytoplasm (Karatzas et al., 2010). GABA itself is less acidic than glutamate and its accumulation in the cell cytoplasm also contributes to an increase in pH (Cotter et al., 2001). Interestingly, the absence of σ^B resulted in no changes in the levels of GABA after exposure to pH 2.5 for 1h (Ferreira et al., 2003), suggesting that while σ^B is involved in regulating the GAD system, there may be an alternative mechanism regulating the production of GABA.

The ADI system increases the cytoplasmic pH through the conversion of arginine to ornithine, and ammonia, with the latest being converted to ammonium through the addition of an intracellular proton (Ryan et al., 2009). Arginine is either synthesised from glutamate via the arginine synthesis pathway, or transported into the cell via the ArcD transporter (Gahan and
Hill, 2014), while its conversion to citrulline is regulated by the ArcA protein encoded by \textit{lmo0043}, which transcription is up-regulated by $\sigma^B$ (Hain et al., 2008).

\textbf{ii. LIGHT STRESS}

The RsbR parologue YtvA of \textit{B. subtilis} was identified as a blue light sensor enclosing a light, oxygen, voltage (LOV) domain upstream from the sulphate transporter and anti-sigma factor antagonist (STAS) domain that shared a high level of homology with plant phototropins (Losi et al., 2002). The RsbR parologue Lmo0799 (homologous to \textit{B. subtilis} YtvA) was identified as a blue light sensing protein (Chan et al., 2013; Ondrusch and Kreft, 2011). Several amino acids required for its functionality were found conserved, including Cys62 and Cys56 in YtvA and Lmo0799, respectively, which is essential for the activation of $\sigma^B$ in response to blue light irradiation, and its alteration to either a serine or alanine residue inhibits $\sigma^B$ activation in response to blue light irradiation (Ávila-Pérez et al., 2006; Gaidenko et al., 2006; O’Donoghue et al., 2016). The LOV domains are able to regulate kinase activity in response to excitation by blue light via their reversible binding of flavin mononucleotide (FMN), the product of the phosphorylation of riboflavin by riboflavin kinase that acts as a chromophore (Losi et al., 2002; Wishart et al., 2018). Upon irradiation with blue light, the carbon C(4a) within the FMN forms a reversible covalent adduct with the conserved cysteine residue of the LOV domain (Christie, 2007), which is critical for the activation of $\sigma^B$ in response to blue light irradiation in both \textit{B. subtilis} and \textit{L. monocytogenes} (Ávila-Pérez et al., 2006; Gaidenko et al., 2006; O’Donoghue et al., 2016; Ondrusch and Kreft, 2011).

The deletion of \textit{lmo0799} from the genome showed no increase in the sensitivity of \textit{L. monocytogenes} to killing by blue light (O’Donoghue et al., 2016), although the induction of $\sigma^B$-dependent genes required Lmo0799 (Tiensuu et al., 2013), which highly suggested that bacteria are able to sense and respond to alternative stresses associated with photodynamic inactivation (PDI) via an alternative sensory mechanism. While $\sigma^B$ was shown to be required for resistance to lethal blue light, its absence was found beneficial for growth in sub-lethal levels (O’Donoghue et al., 2016). In addition, growth inhibition of \textit{L. monocytogenes} by visible light was proposed to be due to reactive oxygen species (ROS) (O’Donoghue et al., 2016);
however, no variations in transcription of the sod or kat genes thought to be involved in tolerance to ROS exposure to blue light were found in L. monocytogenes (Ondrusch and Kreft, 2011).

**iii. Oxidative stress**

Oxidative stress results from an unbalance in the ratio oxidant/antioxidant in the favour of oxidants, leading to destructive consequences for the cell once the antioxidant mechanisms are overcome (Birben et al., 2012). The three most physiologically important categories of ROS include superoxide (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radical (•OH) (Imlay, 2013), that may lead to DNA damage, lipid peroxidation, and oxidative damage of proteins (Bandyopadhyay et al., 1999; Cabiscol et al., 2000). In L. monocytogenes, σB was found to contribute to oxidative stress resistance (Ferreira et al., 2001), with three σB-dependent genes (lmo0515, lmo1580 and lmo2673) being consequentially identified as having a role in the resistance of L. monocytogenes to oxidative stress (Seifart Gomes et al., 2011). As previously mentioned, the miniprotein Prli42 responsible for anchoring RsbR to the cell membrane, was also shown to be required to mediate the activation of σB by oxidative stress (Impens et al., 2017). Curiously, σB was found to be deleterious for stationary phase cells in the presence of hydrogen peroxide (Boura et al., 2016), which raised doubts in the role of σB in oxidative stress tolerance.

**iv. Osmotic stress**

By employing several mechanisms to overcome osmotic stress, such as the uptake of compatible solutes, including glycine betaine, glutamate and carnitine, from the extracellular environment, L. monocytogenes is able to withstand up to 20h exposure to 7 M NaCl, salt concentrations (Liu et al., 2005; O’Byrne and Booth, 2002; Tombras Smith, 1996) The uptake system of glycine betaine, the predominant compatible solute accumulated by L. monocytogenes in response to osmotic stress (Ko et al., 1994), is encoded by betL (Sleator et al., 1999), which has a σB promoter -33 bases upstream (Fraser et al., 2000). In addition, σB
was shown to contribute to glycine betaine accumulation in response to osmotic stress (Fraser et al., 2003). The second most important osmolyte involved in *L. monocytogenes* osmotic stress tolerance is carnitine, which has been shown to be critical for growth and survival in the murine gastrointestinal tract (Sleator and Hill, 2010; Sleator et al., 2001). Carnitine is transported into the cell via the OpuC transport system, encoded by the *opuCA, CB, CC, CD* operon that has a σ^B^-promoter upstream of *opuCA* (Fraser et al., 2000). While the absence of either OpuC or σ^B^ almost completely abolished carnitine uptake (Fraser et al., 2003), the level of OpuCA in exponentially growing cells increased in proportion to the level of osmotic stress encountered by the cells (Utratna et al., 2011).

### V. Motility and Biofilm Formation

*L. monocytogenes* only expresses flagella at temperatures below 37°C (Peel et al., 1988), as motility is repressed at 37°C by MogR binding upstream from a flagellin-encoding gene, *flaA*, inhibiting transcription (Grundling et al., 2004). 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, at temperatures ≤ 30°C (Shen and Higgins, 2006). At higher temperatures, GmaR undergoes a conformational change, inhibiting complex formation with MogR, therefore leaving MogR free to bind to DNA target sites, repressing transcription of motility genes (Kamp and Higgins, 2011).

Similarly, a variable and temperature-dependent role on biofilm formation was attributed to σ^B^ in *L. monocytogenes*, as its activity was constantly shown during biofilm formation under static and continuous flow conditions only at 30°C or lower temperatures, while the absence of σ^B^ resulted on a significant decrease in biofilm formation (Lemon et al., 2010; van der Veen and Abee, 2010b). The loss of flagella inhibited the initial attachment of cells to the surface, but resulted in hyperbiofilm formation when conducted in flow cells (Todhanakasem and Young, 2008). These findings were not consensual among different studies (Lemon et al., 2007). However, cell motility via the flagella was found to be compulsory for biofilm formation in microtitre plates (Lemon et al., 2007; Todhanakasem and Young, 2008). In the absence of σ^B^, the motility repressor, MogR, is not transcribed leading to increased motility gene...
expression (Raengpradub et al., 2008). Therefore, the reduction in biofilm formation in the absence of $\sigma^B$, suggests that either the dysregulation of motility negatively impacts biofilm formation, or that $\sigma^B$ is required for a role other than regulation of motility in biofilm formation.

**vi. Virulence**

A whole transcriptome comparison of gene expression levels in the intestine compared to brain-heart infusion (BHI) broth identified altered expression levels for 1,206 genes, of which 232 were regulated in a $\sigma^B$-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 $\sigma^B$ promoter and a PrfA binding site were altered in a PrfA-dependent but in a $\sigma^B$-independent manner (Toledo-Arana et al., 2009). This alteration in transcriptional regulation indicates a complex overlap between PrfA and $\sigma^B$ in virulence gene expression and suggests a switch between these regulators as the infection progresses beyond the gastrointestinal tract.

The first step in colonising the mammalian host is the successful transit through the extremely acidic conditions encountered in the stomach. $\sigma^B$ 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., 2013). 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 et al., 2009).

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

The opuC operon of L. monocytogenes is involved in the uptake of carnitine and glycine betaine in response to osmotic stress (Sleator et al., 2001). In both L. monocytogenes ScottA and LO28 strains, the inactivation of the opuC operon results in reduced colonisation of the small intestine in the mouse virulence model (Sleator et al., 2001). In addition to opuC, L. monocytogenes also encodes two additional osmolyte transporters in its genome, betL and gbu, however their deletion from the genome does not significantly alter virulence of the organism (Wemekamp-Kamphuis et al., 2004).Unlike the other transporters, OpuC is the only transporter able to transport carnitine, a molecule that is readily available in mammalian cells, suggesting that carnitine is required for L. monocytogenes 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 and Hill, 2014; Sue et al., 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 et al., 2005; Fraser et al., 2003; Sue et al., 2003). Indeed, mutants lacking σ^B are exquisitely sensitive to bile (Begley et al., 2005; Zhang et al., 2011). Interestingly, studies into the requirement of OpuC for virulence identified a bile-sensitive phenotype for the ΔopuC mutant, a phenotype which could be reversed through the addition of exogenous carnitine (Watson et al., 2009). The mechanism behind this observation has not yet been defined but one possibility is that bile might perturb osmoregulation and the compatible solute carnitine could help to mitigate this effect.
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 prfA (Camilli et al., 1993). The other two alternative promoter sites P_{1prfA} and P_{2prfA} are located immediately upstream of prfA (Freitag et al., 1993). The first (P_{1prfA}) is recognized only by the *L. monocytogenes* housekeeping sigma factor σ^A, while P_{2prfA} consists of two overlapping promoters, one recognized by σ^A and the other by σ^B (Nadon et al., 2002). Moreover, the activity of the P_{2prfA} promoter region was shown to contribute to the majority of the prfA transcripts in both intra- and extracellular bacteria (Kazmierczak et al., 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 P_{2prfA} 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 et al., 2009).

### 1.4.3. **Role of σ^B in Saprophytism and in the Food Chain**

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 σ^B regulation as outlined in the previous sections (van der Veen and Abee, 2010b).

#### i. **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 11h simulated days (20°C, 4 UV, and 3 luminescent lights), 13h nights (16°C, no light), and 70% humidity (Gorski et al., 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 et al., 2011). Upregulation of sigB transcription was observed after incubation in piggery lagoon effluent and a high proportion of the σ^B regulon was differentially transcribed (Vivant et al., 2017).

Soil may be a reservoir of *L. monocytogenes* from which transfer to plants may occur (NicAogáin and O'Byrne, 2016; Vivant et al., 2013b). This transfer process apparently requires σ^B activity as transfer of *L. monocytogenes* from contaminated soil to radish was significantly lower when σ^B was not functional (Gorski et al., 2011). In another study, 5h 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, 16h, 25°C) (Rieu et al., 2010). Activity of σ^B is required for chitinase activity. Chitin, an insoluble polymer of N-acetyl-D-glucosamine that is abundant in the biosphere and is degraded by two *L. monocytogenes* chitinases ChiA and ChiB. In the absence of sigB, expression of both *chiA* and *chiB* was reduced (Larsen et al., 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 during life outdoors but the actual niche-specific environmental cues triggering σ^B activity have been poorly investigated to date.

**ii. Foodstuff and Food Production Premises**

Although *L. monocytogenes* is commonly found in the food chain (NicAogáin and O'Byrne, 2016), reports on the activity of σ^B in complex food matrices 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 48h 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 et al., 2010). 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 et al., 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 et al., 2012). Data is available after incubation in fermented sausage, minced meat, soft cheese and ultra-High Temperature milk (Alessandria et al., 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 et al., 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 σ^B^ (Ryan et al., 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 and Abee, 2010a). 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 et al., 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 and 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 et al., 2006; Shin et al., 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.
1.5. **Central role of σ^{B} in *L. monocytogenes* regulatory network: overlaps with other regulators**

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.

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 *et al.*, 2012; Guariglia-Oropeza *et al.*, 2014; Hu *et al.*, 2007; Lobel and 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.

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1.5.1. **AgrA**

AgrA is the transcriptional regulator of the agr communication system in *L. monocytogenes*, which is involved in adhesion to abiotic surfaces and early stages of biofilm formation (Riedel *et al.*, 2009; Rieu *et al.*, 2007), infection of the mammalian host (Autret *et al.*, 2003), and soil
adaptation (Vivant et al., 2015) – revised in section 1.3 of this thesis. 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 σB and Agr systems of S. aureus. While σB increases sar expression, it apparently decreases RNA III production in a growth-phase dependent manner (Bischoff et al., 2001). However, although no direct link between agr and σB has been reported in L. monocytogenes, evidence of synergistic activity of the two systems has been reported. Several genes from the σB regulon showed significant changes in transcript levels between ΔagrA mutant and WT strains at both saprophytic (25°C) and mammalian host (37°C) temperatures in L. monocytogenes EGD-e (Garmyn et al., 2012). Interestingly, some of these genes are co-regulated by PrfA as well.

1.5.2. CodY

Initially discovered in B. subtilis (Slack et al., 1993), the transcriptional repressor CodY is exclusive to low G+C content Gram-positive bacteria, including L. monocytogenes (Geiger and 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 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 and Herskovits, 2016).

The regulatory crosstalk between CodY and PrfA, AgrA and σB 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 et al., 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 four-gene operon that includes sigB (*rsbV*, *rsbW*, *sigB*, *rsbX*), thereby identifying a direct regulatory link between CodY and σ^B_ (Lobel and Herskovits, 2016). Thus, during mammalian infection, conditions in which BCAAs are considered to be limited (Brenner et al., 2018; Lobel et al., 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 et al., 2008), may be repressed in nutrient-rich conditions as a result of σ^B_ repression by CodY (Lobel and Herskovits, 2016).

1.5.3. **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 et al., 2000). CtsR negatively regulates class III stress response genes (Nair et al., 2000). Class III stress response genes are defined as those lacking the highly conserved CIRCE (controlling inverted repeat of chaperone expression) operator sequence (Hecker et al., 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 of the protein where phosphorylated CtsR is a substrate for degradation by the ClpCP complex (Kruger et al., 2001). Transcription of the modulators of CtsR repression (McsA and McsB) is an example of co-regulation by CtsR and
\( \sigma^B \) (Hu et al., 2007). It results in a set of two promoter regions in the mcsA-mcsB-clpC operon, a \( \sigma^B \)-dependent promoter upstream of mcsA and a \( \sigma^A \)-dependent promoter with a CtsR binding site upstream of ctsR. A total of 40 genes are co-regulated by \( \sigma^B \) and CtsR in \textit{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 and Abee, 2010a). A \( \sigma^B \) consensus promoter sequences upstream of the 5' portion of the hrcA-grpE dnaK operon hrcA and dnaK indicates a direct positive regulation of hrcA and dnaK by \( \sigma^B \), and provides evidence for a regulatory network involving these two regulators (Raengpradub et al., 2008). While \( \sigma^B \) 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 \( \sigma^B \), or all three regulators (Hu et al., 2007). Moreover, a total of 37 genes and 30 genes of the \( \sigma^B \) 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 \( \sigma^B \) and the negative regulators CtsR and HrcA is required to shape the heat shock response in \textit{L. monocytogenes}.

### 1.5.4. MogR

The synthesis of flagella in \textit{L. monocytogenes} is regulated by temperature, with higher expression at low temperature, and by the transcriptional repressor of all known flagellar genes, MogR (Shen and Higgins, 2006). Two promoter regions were identified upstream MogR, P1 and P2, sharing a common Rho-independent transcription termination (Toledo-Arana et al., 2009). P1 is a \( \sigma^B \)-dependent promoter, which produces a five gene-long polycistronic mRNA, overlapping three genes required for the synthesis of the \textit{Listeria} flagellum on the opposite strand, \textit{Imo0675}, \textit{Imo0676} and \textit{Imo0677}. On the other hand, P2 produces the bicistronic mRNA morgR-\textit{Imo0673}, which is constitutively expressed. However, the absence of the polycistronic transcript is not sufficient to explain the fact that, at low temperatures, the \( \Delta \text{sigB} \) mutant has increased motility, since the transcription abrogation
from P1 leads to increase of flagellum gene expression but not to an increase in motility (Toledo-Arana et al., 2009).

Repression by MogR is less stringent at low temperatures to allow for flagella production and motility (Grundling et al., 2004). The bifunctional protein GmaR, a glycosyltransferase that has O-linked N-acetylglucosamine transferase activity for flagellin and also acts as a thermostating anti-repressor that incorporates temperature signals into transcriptional control of flagellar motility (Kamp and 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 and Higgins, 2006). In the wild-type transcription of the fliNgmaR 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 transcription of gmaR, 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 and 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 σB.

1.5.5. PrfA

To conserve metabolic resources, L. monocytogenes uses temperature as a signal for sensing the host environment. Although generally not conserved, RNA thermostors are key transcriptional regulators commonly used by pathogenic bacteria for activating virulence genes (Ignatov and Johansson, 2017; Winkler and Breaker, 2003). In L. monocytogenes, the most well-known virulence RNA thermostors 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 thermostensor. 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 et al., 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 et al., 2009).

The overlap between the σB and the PrfA regulons is perhaps one of the most well documented interconnections in L. monocytogenes. The σB-dependent promoter region upstream prfA (P2prfA) is not only accountable for an increase of prfA transcription, but σB itself is also involved in reducing the cytotoxic effects of constitutively active PrfA, suggesting a multilevel regulatory link between σB and PrfA. The overlap between the σB and PrfA regulons has been well described (Chaturongakul et al., 2011; Milohanic et al., 2003; Ollinger et al., 2009). A search for hypothetical σB-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 σA-dependent promoter. However, a second set of PrfA-regulated genes lacking a PrfA box seem to be expressed from a σB-dependent promoter (Milohanic et al., 2003). However, only a total of 11 genes of the σB regulon were found 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 σB promoters and appear to be coregulated by both PrfA and σB, even though contributions of
σ^B and PrfA to *inlA* transcription may be apparent only under specific growth conditions (Kim *et al.*, 2005; McGann *et al.*, 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 *et al.*, 2006). In this context, while trigging stress response and activating virulence, an interconnection between pleiotropic σ^B and the PrfA transcriptional regulators is one important network (Chaturongakul *et al.*, 2011; Ollinger *et al.*, 2009).

### 1.5.6. **ALTERNATIVE SIGMA FACTORS**

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^I) (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 and Karatzas, 2008), homeostasis and resilience (Liu *et al.*, 2017), the regulons controlled by other alternative sigma factors are relatively 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 *et al.*, 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 *et al.*, 2004). The σ^I 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 *et al.*, 2009; Tessema *et al.*, 2012).

Several genes from multiple biological function categories were found coregulated by σ^B, and at least one additional sigma regulator (Mujahid *et al.*, 2013). Of the σ^B regulon, (i) 92 genes were also regulated by σ^H; (ii) 31 genes by σ^I; 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).
absence of phenotypic consequences in the loss of multiple sigma factors suggests functional redundancies among these regulators (Chaturongakul et al., 2011), which largely makes it futile to classify a gene as a member of one specific regulon.

1.5.7. $\sigma^B$-DEPENDENT SMALL REGULATORY RNAs

Small non-coding RNAs 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 non-coding 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 and 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 antisense RNAs (asRNAs) (Becavin et al., 2017).

The transcription of several small regulatory RNAs of *L. monocytogenes* depends on $\sigma^B$ (Table 1.1) and they each possess a $\sigma^B$-dependent promoter upstream of their coding region. This group includes sRNAs of all classes: three trans-acting sRNAs (SbrA, Rli47 and Rli33-1), two antisense RNAs (Anti-LhrC-5 and Anti2270) and a cis-acting regulatory element (Rli95) (Mraheil et al., 2011).

Additionally, $\sigma^B$ can also control sRNAs via the $\sigma^B$-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 et al., 2004). Here, we highlight a selection of $\sigma^B$-dependent sRNAs that contribute to *L. monocytogenes* stress tolerance and adaptation to saprophytic and virulence-specific niches.
Table 1.1. Regulatory small non-coding 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 antisense 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*.

<table>
<thead>
<tr>
<th>σ^B-dependent sRNAs</th>
<th>Targets</th>
<th>Associated Phenotypes and Remarks</th>
<th>References</th>
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<tr>
<td><strong>Trans-acting sRNAs</strong></td>
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<tr>
<td>SbrA</td>
<td>Undetermined.</td>
<td>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.</td>
<td>Nielsen et al., 2008; Oliver et al., 2009; Toledo-Arana et al., 2009.</td>
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<tr>
<td>Rli33-1</td>
<td>oppA (virulence-associated oligo-peptide binding protein), lapB (cell wall anchored virulence adhesion) and tcsA (CD4+ T cell-stimulating antigen) mRNAs.</td>
<td>Unlike its other sibling sRNAs, it is not induced by heme toxicity. Required for <em>L. monocytogenes</em> infection of macrophages. It has a role on bacterial pathogenicity.</td>
<td>Mraheil et al., 2011; Mollerup et al., 2016; dos Santos et al., 2018.</td>
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<tr>
<td>Rli47</td>
<td>Suggested targets: <em>lmo0636</em> and proteins Lmo0637 (methyltransferase) and Lmo2094 (L-fuculose-phosphate aldolase).</td>
<td>Highly expressed in cells on stationary phase, in macrophages, in the intestinal lumen, under oxidative stress and during soil adaptation on a ΔagrA mutant. Possible involvement in virulence and saprophytic lifestyle.</td>
<td>Oliver et al., 2009; Toledo-Arana et al., 2009; Mraheil et al., 2011; Mujahid et al., 2012; Vivant et al., 2015.</td>
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<td><strong>Cis-acting sRNAs</strong></td>
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<td><strong>Antisense sRNAs</strong></td>
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<tr>
<td>Anti-LhrC-5</td>
<td><em>lhrC-5</em> (virulence associated sRNA) and <em>lmo0946</em> (hypothetical protein).</td>
<td>Over-expressed in hypoxia. Coding sequence overlaps the sequences of the sRNA LhrC-5 and <em>lmo0946</em>.</td>
<td>Toledo-Arana et al., 2009; Mraheil et al., 2011.</td>
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<tr>
<td><strong>Hfq-dependent sRNAs</strong></td>
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<tr>
<td>LhrA</td>
<td><em>lmo0302</em> (hypothetical protein), <em>chiA</em> (chitinase).</td>
<td>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 <em>chiA</em> and <em>lmo0302</em>. Negative effect on the chitinolytic activity of <em>L. monocytogenes</em>.</td>
<td>Christiansen et al., 2006; Nielsen et al., 2011.</td>
</tr>
</tbody>
</table>
i. **Trans-acting sRNAs**

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 et al., 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 $\sigma^B$-dependent promoter upstream each of the four $\sigma^B$-dependent sRNAs sbrA-D in *L. monocytogenes* EGD-e genome (Nielsen et al., 2010). Of the four putative sRNAs identified as potentially $\sigma^B$-dependent, $\sigma^B$-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). However, RNA-seq analysis revealed that the transcript levels for this sRNA were not $\sigma^B$-dependent in *L. monocytogenes* 10403S (Oliver et al., 2009; Toledo-Arana 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 et al., 2008).

**Rli47:** Rli47 (also referred to as SbrE) is a ~500 nucleotides long non-coding RNA, 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 $\sigma^B$ regulon after a $\sigma^B$-dependent promoter was identified upstream of the coding sequence. Several studies reported Rli47 as being highly transcribed in stationary phase *L. monocytogenes* cells using RNA-seq (Oliver et al., 2009), tiling microarray study (Toledo-Arana et al., 2009), and quantitative RT-PCR (Mujahid et al., 2012). Rli47 was also found to be highly expressed in stationary phase cells exposed to oxidative stress, although this was not consistent with phenotypic data (Mujahid et al., 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 $\sigma^B$ and the AgrA regulons, since a higher level of Rli47 transcripts was reported in a *L. monocytogenes* EGD-e $\Delta$agrA mutant strain during soil survival (Vivant et al., 2015). Microarray and proteomics experiments identified lower transcript levels of the gene *lmo0636*, and reduced levels of Lmo0637 (methyltransferase) and Lmo2094 (L-fuculose-phosphate aldolase) proteins in the $\Delta$rli47 isogenic mutant (Mujahid et al., 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 $\sigma^B$-dependent sRNA remains unknown.

**Rli33-1:** The LhrC family 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 $\sigma^B$ 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 assert a regulatory function for Rli33-1 in the intracellular environment.

### ii. *Cis-encoded sRNAs*

The *cis*-encoded sRNA Rli95 is the only $\sigma^B$-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 *et al.*, 2017). Its transcriptional termination is induced by the purine analogue 6-N-hydroxylaminopurine, hence preventing the expression of its downstream genes *Imo1885* and *Imo1884* that encode a xanthine phosphoribosyl transferase and a xanthine permease, respectively.

### iii. Antisense RNAs

Antisense 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 *et al.*, 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 $\sigma^B$– 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 $\sigma^B$ promoter and whose coding sequence overlaps the sequences of the sRNA LhrC-5 and *Imo0946* (Mraheil *et al.*, 2011). Similarly, Anti2270 is an antisense sRNA partially encoded in the intergenic region between *Imo2269* and *Imo2270* (competence transcription factor ComK', N terminal), overlapping the 5'-UTR of the last. Furthermore, a putative $\sigma^B$ promoter was identified upstream from its sequence (Mraheil *et al.*, 2011), however its function remains to be discovered.
**IV. INDIRECT $\sigma^B$ REGULATION OF sRNAs EXPRESSION THROUGH THE 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-positives and diverges between species (Bouloc and Repoila, 2016). In *L. monocytogenes*, a $\sigma^B$-regulated promoter located in the *hfq* upstream region explains its $\sigma^B$-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 *et al.*, 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 it 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 *et al.*, 2016).
1.6. **AIMS AND OBJECTIVES**

As part of the European research program List_MAPS (Training and Research in *Listeria monocytogenes* adaption through proteomic and transcriptomic deep sequencing analysis), this thesis was expected to contribute to the understanding of the interconnections between AgrA and $\sigma^B$ regulons in the regulatory network of *L. monocytogenes*, assessing the hierarchy of the cell response under specific environmental conditions and understanding the cellular integration of biotic stimuli and harsh conditions. In order to investigate (i) the regulatory circuitry of this pathogenic bacterium through the investigation of the crosstalk between the cell communication system Agr and the stress response mediated by $\sigma^B$; (ii) how adaptation of *L. monocytogenes* is influenced by environmental conditions in soil, plants and biofilms; and (iii) the biological role of the small regulatory RNA Rli47, part of both AgrA and $\sigma^B$ regulons, a set of deletion mutants was constructed to inactivate either cell communication, general stress response or both systems. The phenotypes of these mutants were characterized under several environmental conditions. Furthermore, reporter strains were constructed in order to assess activation of Agr and $\sigma^B$ at the single cell level, under several stress conditions. Moreover, a mutant was obtained in which the intergenic region coding the $\sigma^B$-dependent small regulatory RNA Rli47 was deleted. Understanding Rli47 biological function in the lifestyle of *L. monocytogenes* was set as a purpose.

Overall, results are presented in three results chapters (Chapters 2, 3 and 4). Chapter 2 investigates the contribution of AgrA and $\sigma^B$ to soil fitness and survival of *L. monocytogenes* in response to background microbiota, as well as root colonisation. Chapter 3 focusses on the role of the communication system Agr and the stress response factor $\sigma^B$ during sessile growth of *L. monocytogenes* under sub-lethal osmotic stress conditions. Finally, chapter 4 concentrates on the structure and biological role of Rli47.
CHAPTER 2

INVESTIGATION ON THE ROLES OF AgrA AND σ^B REGULATORS IN Listeria monocytogenes ADAPTATION TO THE OUTDOORS ENVIRONMENT

Catarina M. Marinho, Dominique Garmyn, Laurent Gal, Maja Brunhede, Conor O’Byrne and Pascal Piveteau. Investigation on the roles of AgrA and σ^B regulators in Listeria monocytogenes adaptation to the outdoors environment. Accepted for publication in FEMS Microbiology Letters.
2.1. **PREAMBLE**

The bibliographical study presented in the previous chapter has made it possible to summarize the main information relating to the ecology of *L. monocytogenes* in the outdoors environment. To cope with and survive in the telluric environment, *L. monocytogenes* must be able to integrate environmental signals and respond in a concerted manner to adjust its physiology.

As described in the introduction, the Agr system of *L. monocytogenes* is involved in adhesion to abiotic surfaces (Rieu *et al.*, 2007), in the early stages of biofilm formation (Riedel *et al.*, 2009; Rieu *et al.*, 2007; Zetzmann *et al.*, 2019), soil adaptation (Vivant *et al.*, 2015; Vivant *et al.*, 2014), and host infection (Autret *et al.*, 2003; Riedel *et al.*, 2009). Despite mediating the stress response to a panoply of stresses such as acid (Davis *et al.*, 1996; Ferreira *et al.*, 2001; Ferreira *et al.*, 2003; O 'Driscoll *et al.*, 1996) light (O'Donoghue *et al.*, 2016; Tiensuu *et al.*, 2013), oxidative (Boura *et al.*, 2016; Ferreira *et al.*, 2001; Impens *et al.*, 2017) and osmotic stresses (Fraser *et al.*, 2000; Fraser *et al.*, 2003; Utratna *et al.*, 2011), σ^B^ regulation does also influence biofilm formation (Lemon *et al.*, 2010; Raengpradub *et al.*, 2008; van der Veen and Abee, 2010b), virulence (Begley *et al.*, 2005; Gahan and Hill, 2014; Kazmierczak *et al.*, 2003; Kazmierczak *et al.*, 2006; Kim *et al.*, 2005; Ollinger *et al.*, 2009; Sue *et al.*, 2003; Sue *et al.*, 2004; Toledo-Arana *et al.*, 2009; Zhang *et al.*, 2011), and cell transfer from contaminated soil to plants (Gorski *et al.*, 2011). Evidences suggested a crosstalk between these two systems in *L. monocytogenes* (Garmyn *et al.*, 2012). Although both systems independently seem to contribute to *L. monocytogenes* saprophytic lifestyle, the nature of the interconnection between AgrA and σ^B^ in the outdoors environment is not yet described. This chapter will therefore be devoted to assess the contribution of these two regulators of *L. monocytogenes* to root colonisation and soil fitness.

Thus, in order to better understand the role of the AgrA and the σ^B^ regulators during the adaptation of *L. monocytogenes* to soil, we studied the evolution of the parental strain and the deletion mutants ΔagrA, ΔsigB and ΔagrAΔsigB inoculated into soil microcosms, in the presence or absence of the native soil microbiota, and in *vitro* roots colonization. Then, a differential transcriptomic approach was used to evaluate the impact of inactivation of one regulator on transcription of the other during *L. monocytogenes* adaptation in soil.
2.2. ARTICLE

**Research Letter**

Investigation on the roles of AgrA and σB regulators in *Listeria monocytogenes* adaptation to the outdoors environment

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**Abstract**

The foodborne pathogen *Listeria monocytogenes* is ubiquitous in the environment and commonly associated with soil and decaying vegetation. Little is known about the regulatory mechanisms that ensure its survival in the telluric environment. Earlier studies have suggested a regulatory overlap between the Agr cell-cell communication system and the general stress response regulator σ^B_. Here, we investigated the contribution of these two systems to survival in sterilised and biotic soil. In sterile soil, growth was observed and similar population dynamics were shown in the parental strain, ΔsigB and ΔagrA mutants. However, a significant defect was observed in the double knockout (ΔagrAΔsigB) mutant, suggesting some synergy between these systems. In biotic soil microcosms, viability of the parental strain declined steadily over a two-week period highlighting the challenging nature of live soil environments. The inactivation of the two systems further affected survival. The synergistic effect of σ^B and Agr was even stronger in biotic soil microcosms. Transcriptional analysis confirmed the expected effects of the mutations on known Agr- and σ^B-dependent genes. The ability to colonise the roots was also significantly compromised in the double mutant. Data highlight the important role that these global regulatory systems play in the natural ecology of this pathogen.

**One-sentence summary**

Synergistic effect of AgrA and σ^B is suggested during *Listeria monocytogenes* soil survival and root colonisation.

**Keywords**

Soil survival, roots colonization, AgrA, σ^B, *Listeria monocytogenes*, transcription regulators
INTRODUCTION

Listeria monocytogenes is a robust foodborne pathogen capable of surviving in a wide range of niches outside the host (O'Byrne & Karatzas, 2008, NicAogáin & O'Byrne, 2016, Dorey, et al., 2019). Soil is a natural reservoir of L. monocytogenes and transfer from contaminated sources such as sewage, animal manure and decaying plant vegetation to soil was observed (Welshimer, 1960, Welshimer & Donker-Voet, 1971, Fenlon, et al., 1996, Nightingale, et al., 2004, Locatelli, et al., 2013, Strawn, et al., 2013, Vivant, et al., 2013). However, populations of L. monocytogenes in bulk soil are generally low (MacGowan, et al., 1994, Dowe, et al., 1997), with an estimated prevalence of less than 10⁴ per gram of dry soil throughout the French territory (Locatelli, et al., 2013). L. monocytogenes can persist in soil over a period of time (Ivanek, et al., 2009, McLaughlin, et al., 2011, Locatelli, et al., 2013). Soil chemical properties and natural microbiota as well as geographical and meteorological conditions may affect survival and persistence of L. monocytogenes (Ivanek, et al., 2009, McLaughlin, et al., 2011, Strawn, et al., 2013). The presence of L. monocytogenes in crop production has often been reported (Brandl, 2006), since it can persist on the surface of plants throughout the cultivation period between 10⁵ and 10⁶ CFU per gram of soil (Jablonske, et al., 2005). Nonetheless, in comparison to in vivo conditions, little is known about the regulatory mechanisms that underlie survival of this pathogen over its saprophytic lifestyle (Dorey, et al., 2019).

The Agr cell-cell communication system of L. monocytogenes is required for optimal adaptation and survival in soil (Piveteau, et al., 2011, Vivant, et al., 2014). The Agr system was reported to activate chitin hydrolysis through repression of LhrA transcription, a small RNA (sRNA) known to repress ChiA translation (Paspaliari, et al., 2014). ChiA and ChiB belong to the chitinolytic system of L. monocytogenes that allows degradation of this second most abundant carbohydrate in nature, and its use as nutrient source (Gooday, 1990, Gooday, 1990, Beier & Bertilsson, 2013). Likewise, σ⁸ does also contribute for the production of chitinases, as its absence results in a reduction in ChiA and ChiB expression and consequent decrease in L. monocytogenes chitinolytic activity (Larsen, et al., 2010). Differential transcriptome analyses during adaptation to biotic soil environments showed large transcriptome alterations when AgrA was not functional, over 500 protein-coding genes
involved in cell envelope and cellular processes, and an extensive repertoire of sRNAs were differentially transcribed (Vivant, et al., 2015). Comparatively, transcriptomic differences under sterilized soil conditions were limited, suggesting that activation of the response regulator AgrA is required for optimal re-shaping of the transcriptional landscape during saprophytic life when L. monocytogenes encounters complex biotic conditions (Vivant, et al., 2015).

The σ^B factor controls the general stress response, coordinating transcription of almost 300 genes (approximately 10% of the genome of L. monocytogenes) (Chaturongakul, et al., 2011). Studies concerning the role of σ^B during L. monocytogenes life in outdoors environments are scarce. The σ^B system is expected to contribute to resistance to stresses that this saprophytic pathogen may encounter in the telluric environment (Dorey, et al., 2019). For example, several genes from the σ^B regulon are differentially transcribed during incubation in soil extracts (Piveteau, et al., 2011). In synthetic soil, inactivation of σ^B resulted in a growth defect of L. monocytogenes and it negatively influenced the ability of the pathogen to transfer from contaminated soil to crops (Gorski, et al., 2011).

Direct link between Agr and σ^B systems was reported in the species Staphylococcus aureus, where σ^B decreases transcription of RNAIII, the regulatory RNA effector of the Agr system (Bischoff, et al., 2001). Although no direct association between these two systems has been reported in L. monocytogenes, differential transcription of several genes of the σ^B regulon when AgrA is not functional suggests crosstalk between the two systems (Garmyn, et al., 2012). The biological function of the σ^B-dependent sRNA Rli47 is yet undetermined (Mujahid, et al., 2012), however its involvement in adaptation to environmental conditions has been suggested, perhaps involving crosstalk between σ^B and AgrA regulons, since higher levels of Rli47 transcripts were reported in a ΔagrA L. monocytogenes EGD-e mutant during soil survival (Vivant, et al., 2015). In this study, we investigated the contribution of the general stress response (σ^B) and cell-cell communication system (AgrA) of L. monocytogenes to root colonisation and soil fitness with deletion mutants. The response to the extrinsic factors “soil microbiota” and “water content” were also assessed.
MATERIALS AND METHODS

Bacterial strains, plasmids, primers and culture media

*Listeria monocytogenes* EGD-e (Glaser *et al.*, 2001) was used as parental strain. The ΔagrA (Rieu *et al.*, 2007) and ΔsigB (Marinho *et al.*, 2018) strains were also used in this study (Table 2.1). A two-step integration/excision procedure based on the mutagenesis pMAD vector (Arnaud *et al.*, 2004) was used to construct the ΔagrAΔsigB in-frame deletion mutant. For this, the deletion of sigB was created in the ΔagrA mutant (DG125A) background. All primers and plasmids used for this construction are listed in Table 2.1. Cloning was performed in One Shot TOP10 Chemically Competent *Escherichia coli* (Invitrogen). Electro-competent cells of *L. monocytogenes* were prepared and transformed as stated by Monk *et al.* (2008). Gene deletions were checked by sequencing PCR products using gene-flanking primers. Whole-genome sequencing was performed by Illumina sequencing (MicrobesNG) and genomes were analysed by Breseq (Deatherage & Barrick, 2014). Sequencing indicated additional amino acid changing point mutations in coding regions of the deletion mutants relatively to the parental strain: one point mutation in the agrA deletion mutant, *lmo1950* (G369→T), and a total of eleven point mutations in the double mutant (ΔagrAΔsigB), *rpsR* (G8→A), *lmo0178* (C964→T), *prs* (C763→A), *ldh* (G548→T), *sigH* (G507→T), *lmo0748* (T230→C), *lmo1066* (C407→A), *lmo1444* (C31→T), *lmo2003* (G130→A), *lmo2360* (C898→A), and *lmo2684* (C962→T). Isogenic rifampicin resistant (RIF) strains EGD-e L9 and DG125A6 were used in this study (Vivant *et al.*, 2014). Transduction of φLMUP35 - EGD-e L9 preparations (Hodgson, 2000) in all other deletion mutants was implemented to transfer rifampicin resistance (RIF) to strains; transformant cells were selected in TSA+RIF 200 μg.ml⁻¹ (Sigma-Aldrich). Rifampicin resistant strains were isolated on Trypton Soy Broth (TSB) (CONDA) and Polymyxin-Acriflavine-Lithium-Chloride-Ceftazidime-Aesculin-Mannitol (PALCAM) agar medium (AES Chemnex) supplemented with 200 μg.ml⁻¹ rifampicin (Sigma-Aldrich) according to Lemunier *et al.* (2005). All strains used in this study are listed in Table 2.1.
### STRAINS

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### PRIMERS

#### Cloning

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</table>
Root colonization kinetics

Seeds of *Festuca arundinacea* were disinfected by washing with 3% sodium hypochlorite at 56°C for 1h, rinsed three times with sterile water and dried at RT. The efficacy of disinfection was assessed by incubating seeds on TSA plates at 25°C. Before use, the percentage of germination of dry disinfected seeds was checked in white agar plates incubated at 30°C for three to four days. Disinfected seeds were germinated in white agar plates incubated at 30°C for three to four days. Seedlings were aseptically transferred to Hoagland mineral solid medium with 5 seedlings per agar plate. All Hoagland plates were incubated in a climatic chamber under cycles of 16h light at 23°C and 8h dark at 18°C for one week. Bacterial inocula were harvested to exponential phase (OD_{600} = 0.4) in TSB medium at 25°C and processed as described above. Each root system was inoculated with 200 µl of inoculum adjusted to 5x10^6 CFU.ml^{-1}. Inoculated Hoagland plates were then incubated in the climatic chamber and the population of *L. monocytogenes* was enumerated over one week (on days 0, 1, 2, 4 and 7 after inoculation). At each sampling time, one plate containing five inoculated seedlings was used per condition. The root system of the five plants was aseptically dissected, weighted and transferred into a vial with 10 ml of TS buffer. A total of 0.7 g of sterile glass beads (≥106 µM acid washed glass beads, Sigma-Aldrich) was added and the vials were vortex for 2 min to remove bacteria from the root. The dynamics of the *L. monocytogenes* populations in the roots were followed immediately after inoculation and periodically over a seven-day period by serial plating on TSA plates.

Preparation of Soil microcosms and *L. monocytogenes* kinetics

Soil was collected at an experimental farming unit in France (Epoisses, (47° 30' 22.1832" N, 4° 10'26.4648''). It is a clay loamy soil with a pH of 7.15. Five soil cores (0-20 cm) were sampled from three location 20 m apart and pooled in a composite sample. Soils were sieved to 0.4 mm. Aliquots were sterilised by γ-radiation (45 kGy; Ionisos, Dagneux). Sterilised and non-sterile soil microcosms were prepared. For each soil sample, triplicate microcosms were prepared by adding 50 g of soil to each of three sterile 180 ml capped plastic tubes.
Bacterial cultures were grown in TSB at 25°C until exponential phase (OD$_{600} = 0.4$) or early stationary phase (OD$_{600} = 1.0$) under static conditions in the dark. The inoculum was washed twice by centrifuging (7,000 rcf, 10 min, RT) and suspending the cell pellet in deionised water. The concentration of these inoculums was estimated by OD$_{600}$ reading. Each soil microcosm was inoculated to achieve a final concentration of 2x10$^5$ CFU.g$^{-1}$ of soil. The volume of the inoculum was adjusted in order to reach a final soil moisture content of 40% or 60% of the water-holding capacity (WHC). After inoculation, soils were stirred with sterile spatula, and incubated in the dark at 25°C for two weeks. The dynamics of *L. monocytogenes* populations in soil were followed immediately after inoculation and at suitable intervals over 14 days. Microcosms were sampled; 1 g (equivalent dry soil) was added to 9 ml TS-buffer (1% Tryptone, 8.5% NaCl) with 0.7 g of sterile glass beads (≥106 µM acid washed glass beads, Sigma-Aldrich) and vortexed for 2 min. The mixture was then serially diluted and numerated on selective PALCAM agar supplemented with 100 µg.ml$^{-1}$ cycloheximide and 100 µg.ml$^{-1}$ rifampicin.

**RNA extraction from soil**

Two hours after inoculation in soil, cell lysis was performed by adding 2 g of inoculated soil in a sterile lysis tube containing 4 g of 106 µM beads, 5 g 1.4 mm ceramic beads, 8x 4 mm glass beads. A total of 500 µl of RNAprotect (Qiagen) and 3.5 ml lysis buffer (2 g casein, 20 ml 1 M sodium phosphate (pH 8), 2 ml 5M NaCl, 10 ml 0.5M EDTA (pH 8)) was added and tubes were homogenised in the FastPrep instrument (MP biomedicals) 4 x 30 s setting of 6 m/s. Samples were incubated for 5 min on ice in between runs. Tubes were then incubated for 15 min at 70°C and centrifuged at 12,000 g for 5 min at 4°C. Aqueous phase was transferred to sterile tubes. For RNA purification, 850 µl phenol-chloroform-isoamyl alcohol (125:24:1, pH = 4.5) was added to each tube and homogenized. Tubes were centrifuged at 14,000 g for 5 min at 4°C and the aqueous phase was transferred to a new tube. The phenol-chloroform-isoamyl alcohol extraction step was repeated. A total of 800 µl chloroform was added to the clear supernatant and homogenized. Tubes were centrifuged at 14,000 g for 5 min at 4°C and the aqueous supernatant transferred to a new tube. RNA was precipitated by adding 1 ml of precipitation solution (30% polyethylene glycol 6,000, 1.6 M NaCl) and incubated at 80°C
overnight. Samples were centrifuged for 30 min at 16,000 g at 4°C and the supernatant aspirated. A total of 800 µl of ice cold 75% ethanol was added to the pellet and mixed well. Samples were centrifuged full speed for 10 min at 4°C. Ethanol was aspirated and pellets were left to dry at RT for 30 min. Dry pellets were then dissolved and pooled in 200 µl nuclease free water. Samples were further purified using Zymobiomics RNA mini kit (Zymo Research). The quantity and quality of extracted RNA was measured by NanoDrop (Thermo Fisher Scientific) and agarose gel electrophoresis.

**cDNA synthesis and Reverse Transcriptase-Quantitative Polymerase Chain Reaction (RT-qPCR)**

Fifty micrograms of total RNA were DNase-treated with TURBO DNA-free kit according to the manufacturer instructions (Invitrogen). First-strand cDNA from purified total RNA was synthesized using SuperScript IV First-Strand Synthesis System (Invitrogen). The cDNA quantity was determined by Qubit fluorometer (Invitrogen) following manufacturer’s recommendations. Three sets of cDNA were analysed, originating from three sets of RNA extracted from triplicate cultures as described above. RT-qPCR was performed using the QuantiTect SYBR Green PCR Kit (Qiagen) and specific primer sets designed for *rli47, ilvA, opuCA* and *drm* (Table 2.1). Primers efficiency was calculated prior to sample runs. Samples were run on a LightCycler 480 System (Roche) with an initial step at 95°C for 15 min, 45 cycles of 15 s at 95°C, 15 s at 53°C and 30 s at 72°C, a melting curve was drawn for 5 s at 95°C, 1 min at 55°C followed by increases of 0.11°C.s⁻¹ until 95°C, and a cooling for 30 s at 40°C. Cycle quantification values were calculated by the software LightCycler 480 Software version 1.5.1 (Roche) and the Pfaffl relative expression formula (Pfaffl, 2001, Pfaffl, *et al.*, 2002). Relative expression ratio was used to analyse RT-qPCR results with *drm* (phosphopentomutase) as reference gene (Rieu, *et al.*, 2007). The experiment was carried out in three biological replicates, each in technical triplicates.
**Statistical analysis**

All experiments were performed using both technical and biological triplicates. The significance of the differences in patterns of survival of listerial populations were assessed by a Two-way ANOVA with Sidak’s multiple comparisons test in both sterilized and biotic microcosms. The same test was used to estimate whether or not the differences in gene transcript levels were statistically different between mutants and the parental strain. Statistical differences between inocula, growth phases and soil water content were analysed by paired Student t-tests.

**RESULTS AND DISCUSSION**

**Deletion of agrA and sigB resulted in a synergistic reduction on L. monocytogenes ability to colonise roots**

To determine if alterations of either the Agr communication system, the general stress response regulator σB, or both affect L. monocytogenes ability to colonise roots, a seven-days *in vitro* study was conducted in roots of *F. arundinacea* after inoculation with stationary phase cells of L. monocytogenes EGD-e, single deletion mutants ΔagrA, ΔsigB and a double deletion mutant ΔagrAΔsigB. The population of the parental strain colonising roots increased within the first day of incubation and stabilised at 10⁶ CFU.root⁻¹ until the end of the experiment (Fig. 2.1). These results are consistent with reports on the ability of *L. monocytogenes* to colonise plants from the plant model *Arabidopsis thaliana* (Milillo, *et al.*, 2008), * Hordeum vulgare* (Kutter, *et al.*, 2006) to several vegetable types (Gorski, *et al.*, 2004, Jablasone, *et al.*, 2005, Gorski, *et al.*, 2008) and during carrot root surface colonisation (Kljujev, *et al.*, 2018). Contaminated plant material is a route of entry of the human pathogen *L. monocytogenes* in the food chain, from where it can ultimately reach the consumer (NicAógáin & O’Byrne, 2016). Although it is expected that ingestion of large doses of *L. monocytogenes* (~10⁶ CFU) is required to cause infection, the total population recorded at the end of incubation is
consistent with the possibility that plants could act as vectors of *L. monocytogenes* and the reported number of listeriosis outbreaks caused by consumption of contaminated fresh products (McLaughlin, *et al.*, 2011).

Figure 2.1. *L. monocytogenes* colonization of roots of *Festuca arundinacea*. Logarithmic values of CFU per root on the mutant strains are presented. Each coloured line represents the behaviour profile of each strain, parental (blue), ΔagrA (red), ΔsigB (purple) and ΔagrAΔsigB (green) mutants. The data represents three biological replicates (n=15 plants) with three technical independent repetitions. Error bars indicate standard deviation. Asterisks represent *p*-values (* = *p*-value <0.05, ** = *p*-value <0.001, *** = *p*-value <0.0001) calculated using a Two-way ANOVA with Sidak’s multiple comparisons test.

Deletion of either *agrA* or *sigB* did not significantly affect growth of *L. monocytogenes* at the surface of the roots suggesting that inactivation of the Agr or σ^B^ systems did not affect the initial growth of the population, however significant differences between the ΔsigB deletion mutant and parental strain were observed at day 7 (Fig. 2.1), suggesting a role for σ^B^ once the population has settled on the root. Under laboratory conditions, impaired attachment and colonisation on radish tissue when σ^B^ was inactivated have been reported previously (Gorski, *et al.*, 2011). Results with the double mutant strain were different. After the initial growth during the first day of incubation, the population of ΔagrAΔsigB mutant declined until the end of the experiment, and finally dropped back to its initial level (Fig. 2.1). This additive effect of
agrA and sigB deletions suggested synergy between the two systems during colonisation by *L. monocytogenes* of the roots.

*L. monocytogenes* populations increased in sterilised soil, but growth was affected by the double mutation

Sterilised soil microcosms were inoculated with exponential phase cells of *L. monocytogenes* strains as this was the most commonly used inoculum condition in previous similar studies (Vivant, *et al.*, 2013, Vivant, *et al.*, 2014, Vivant, *et al.*, 2015). In sterilised soil microcosms, the population of the parental strain increased over the first day of incubation and the population remained stable until the end of the experiment (Fig. 2.2). These results are in agreement with previous reports on the ability of *L. monocytogenes* to thrive in sterilised soil as a result of inactivation of microbial communities (Dowe, *et al.*, 1997, Moshtaghi, *et al.*, 2009, McLaughlin, *et al.*, 2011, Piveteau, *et al.*, 2011, Locatelli, *et al.*, 2013, Vivant, *et al.*, 2013, Vivant, *et al.*, 2014). However, the water content affected the final level of the population and higher maximal populations were recorder at 60% WHC.

![Figure 2.2. Growth kinetics of *L. monocytogenes* strains in sterilized soil microcosms. Logarithmic values of CFU per gram of soil are presented. Each coloured line represents the behaviour profile of each strain, parental (blue), ΔagrA (red), ΔsigB (purple) and ΔagrAΔsigB (green) mutants. The data represents three biological replicates with three technical independent repetitions. Error bars indicate](image-url)

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standard deviation. Asterisks represent \( p \)-values (* = \( p \)-value <0.05, ** = \( p \)-value <0.001, *** = \( p \)-value <0.0001) calculated using a Two-way ANOVA with Sidak’s multiple comparisons test.

Deletion of either \( \text{agrA} \) or \( \text{sigB} \) did not affect growth in sterilised soil at 40\% WHC but differences between the \( \Delta \text{agrA}\Delta \text{sigB} \) and the parental populations were highly significant from day 1 until the end of incubation (\( p \)-value < 0.0001). When the water content was increased to 60\%, this phenotype was limited and again, similar growth kinetics of the single mutants and the parental strain were observed. These results suggested that despite the fact that inactivation of either the Agr system or the general stress response did not affect the dynamics of \( \text{L. monocytogenes} \) population, the simultaneous loss of the two systems resulted in impaired growth, especially when the water content of sterilised soil was low.

In biotic soil, functional AgrA and \( \sigma^B \) are required for maximum survival of \( \text{L. monocytogenes} \) and further growth defect was recorded after simultaneous inactivation of the two systems.

The presence of indigenous microflora, in biotic soil microcosms, resulted in different population dynamics (Fig. 2.3). Firstly, no growth was observed in any strain and under any of the experimental conditions tested. The population of the parental strain was stable during the first two days of incubation, although it declined dramatically thereafter (4 log). The deletion of \( \text{sigB} \) affected survival of both exponentially grown and stationary phase cells under every condition tested (Fig. 2.3), even if induction of \( \sigma^B \) activity is known to occur during transition to stationary phase (Utratna, \textit{et al.}, 2012). This highlighted a beneficial contribution of the \( \sigma^B \) system to \( \text{L. monocytogenes} \) soil survival. Similar results were observed in the \( \Delta \text{agrA} \) mutant population, with the inactivation of the Agr system resulting in decreased \( \text{L. monocytogenes} \) populations in biotic soil, in all conditions tested (Fig. 2.3)
Figure 2.3. Dynamics of (A) exponential and (B) stationary growth phase \textit{L. monocytogenes} populations in biotic soil microcosms at 40\% and 60\% of total water holding capacity. Logarithmic values of CFU per gram of soil are presented. Each coloured line represents the behaviour profile of each strain, parental (blue), \(\Delta agrA\) (red), \(\Delta sigB\) (purple) and \(\Delta agrA\Delta sigB\) (green) mutants. The data represents three biological replicates with three technical independent repetitions. Error bars indicate standard deviation. Asterisks represent \(p\)-values (* = \(p\)-value <0.05, ** = \(p\)-value <0.001, *** = \(p\)-value <0.0001) calculated using a Two-way ANOVA with Sidak’s multiple comparisons test.

A dramatic decline of the population of the double mutant was observed, especially at 40\% WHC and with exponential phase inocula (Fig. 2.3 A). Interestingly, despite the significant decline in survival of the \(\Delta agrA\) and \(\Delta sigB\) mutant populations relative to the parental, this sharper reduction in the double mutant population throughout the duration of the experiment suggested a that some synergy might be occurring between these two systems during soil survival. Differences in population dynamics according to the water content were not significant in biotic soil (Table 2.2). Likewise, cells growth phase did not impact \textit{L.}
monocytogenes soil survival in any of the water contents tested (Table 2.2), except that stationary phase cells of the parental population showed a more resilient phenotype in soil at 40% TWHC than exponentially grown cells over the first seven days of this experiment (Fig. 2.3 A and B). Soil moisture might affect microbial communities by controlling available oxygen, substrates and water in the soil pore space. The conclusions of several studies on the effect of soil water content on the microbial community are not unanimous. While some suggested that water content is a major determinant of the soil’s microbial community composition and biomass (Borowik & J., 2016, Li, et al., 2017), other studies reported that soil moisture does not affect soil microbial community (Bossio & Scow, 1995, Gordon, et al., 2008, Wu, et al., 2010).

Table 2.2. Statistical analysis of differences in strain survival resulted by growth phase and soil’s water content. Differences were calculated using paired t tests (t = t-value; df = degrees of freedom; * = p-value <0.05). TWHC, Soil’s total water holding capacity.

<table>
<thead>
<tr>
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<th>TWHC = 40%</th>
<th>TWHC = 60%</th>
<th>Soil’s water content</th>
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<td>Exponential phase</td>
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<td></td>
<td>Exponential phase</td>
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<tr>
<td>Paired t test</td>
<td>t</td>
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<tr>
<td>Parental</td>
<td>2.804</td>
<td>5</td>
<td>0.03780 *</td>
</tr>
<tr>
<td>∆agrA</td>
<td>1.574</td>
<td>5</td>
<td>0.1764</td>
</tr>
<tr>
<td>∆agrA∆sigB</td>
<td>0.5313</td>
<td>5</td>
<td>0.6179</td>
</tr>
<tr>
<td>∆sigB</td>
<td>1.356</td>
<td>5</td>
<td>0.2331</td>
</tr>
</tbody>
</table>

Previous studies showed that impairment of AgrA reduced adaptation of L. monocytogenes to the soil environment (Vivant, et al., 2014, Vivant, et al., 2015). The AgrA regulon includes genes responsible for the transport and metabolism of amino acids, motility and chemotaxis, and also genes that code for regulators (Garmyn, et al., 2012). In the biotic soil environment, over 500 protein-coding genes involved in cell envelope, cellular processes, and resistance to antimicrobial peptides, as well as an extensive repertoire of sRNAs were differentially

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transcribed (Vivant, et al., 2015). Together with our results, these studies provide evidence that activation of the Agr system may be critical when _L. monocytogenes_ has to face unfavourable conditions in the telluric environment. Moreover, $\sigma^B$ activity benefits _L. monocytogenes_ resistance to stresses such as osmotic, acid and oxidative stresses (NicAogáin & O'Byrne, 2016, Dorey, et al., 2019). Data suggest a contribution of the general stress response to face unfavourable conditions that _L. monocytogenes_ may encounter in soil. The present data dealing with soil collected from a field confirm previous results from commercial potting soil experiments (Gorski et al. 2011).

It is striking that these unfavourable conditions are mainly microbiome driven as $\sigma^B$ inactivation was detrimental only in live soil. This confirms the role of soil’s endogenous microbial communities in limiting colonisation by _L. monocytogenes_ (Dowe, et al., 1997, Moshtaghi, et al., 2009, McLaughlin, et al., 2011, Vivant, et al., 2013, Vivant, et al., 2014). Under these conditions, _L. monocytogenes_ faces competition and/or antibiosis generated by the activity of fungi (Takahashi, et al., 2008, Bigwood, et al., 2012), bacteria (Sharma, et al., 2014, Olanya & Lakshman, 2015, Undabarrena, et al., 2016, Helfrich, et al., 2018) and other components of the soil microbiome. Abundance, diversity and structure of microbial communities are key parameters of these competitions (Vivant, et al., 2013). Soil protozoa and nematodes could facilitate survival of _L. monocytogenes_ by adding to dispersion of the pathogen (Vivant, et al., 2013). Nonetheless, antimicrobial (Undabarrena, et al., 2016, Helfrich, et al., 2018) and bacteriocin-like compounds (Sharma, et al., 2014) produced by autochthonous bacteria, as well as the presence of predatory microorganisms (Olanya & Lakshman, 2015) are inhibitory to _L. monocytogenes_, preventing it from thriving in most outdoor environments. Taken together, our results suggest that there might be functional redundancy in the regulation of these two key mechanisms and loss of one could be compensated to some extent by the other in the process of adjusting the population’s physiology to environmental conditions. It suggests both AgrA and $\sigma^B$ cooperatively modulate _L. monocytogenes_ transcriptome to achieve the right balance of adaptations necessary for growth and survival. However, when both regulators are impaired, _L. monocytogenes_ may fail to adapt appropriately.
While in sterile soil σB inhibits agrA transcript levels, agrA transcription is restored in the biotic microcosms

Transcript levels of rli47, sigB, agrA and opuCA were assessed in L. monocytogenes parental, ΔagrA, ΔsigB and ΔagrAΔsigB mutant strains during incubation in sterilized and biotic soil, and analysed relatively to the parental strain (Fig. 2.4). As expected, no sigB or agrA transcripts were detected in the ΔsigB and ΔagrA mutants, respectively, nor both in the ΔagrAΔsigB mutant. Significantly lower relative transcript levels of rli47 and opuCA were found in the ΔsigB and ΔagrAΔsigB mutants, thus confirming the σB-dependent transcription of these genes (Toledo-Arana, et al., 2009).

No significant differences were recorded in the relative transcript levels of rli47 in the ΔagrA mutant during adaptation to sterilised and biotic soil. In a previous study, the transcript levels of rli47 were found higher in the ΔagrA background than in the parental strain during adaptation to the soil environment (Vivant, et al., 2015). Altogether, these data lead to uncertainty on the role of the sRNA Rli47 in L. monocytogenes soil adaptation. Significantly lower relative transcript levels of opuCA were found in the ΔagrA background during adaptation to the soil environment. This phenomenon was previously reported by Garmyn et al. (2012), where a decrease in the relative transcripts of the opuCABCD operon was found when AgrA was not functional.
Figure 2.4. Impact of sigB and/or agrA deletion on rli47, sigB, agrA and opuCA transcript levels in (A) sterilised and (B) biotic soil. Quantification of rli47, sigB, agrA and opuCA was accessed in the ΔagrA, ΔagrAΔsigB and ΔsigB populations relative to the parental strain by RT-qPCR and normalized to drm. Each coloured bar represents the normalised gene expression of each mutant strain, ΔagrA (red), ΔsigB (purple) and ΔagrAΔsigB (green) relative to the parental strain. Logarithmic fold change values on the mutant strains are relative to the parental strain. The data represents three biological replicates with three technical independent repetitions. Error bars indicate standard deviation. Asterisks represent p-values (* = p-value < 0.05, ** = p-value < 0.001, *** = p-value < 0.0001) calculated using a Two-way ANOVA with Sidak’s multiple comparisons test.
During adaptation to sterilized soil, relative transcript levels of \textit{agrA} (log$_2$FC = 5.0 ± 0.30, \textit{p}-value < 0.0001) and \textit{sigB} (log$_2$FC = 0.82 ± 0.33, \textit{p}-value ≥ 0.05) were increased in the \textit{ΔsigB} and \textit{ΔagrA} populations, respectively (Fig. 2.4 A). However, during adaptation to biotic soil, these differences were not significant (\textit{p}-value ≥ 0.05) (Fig. 2.4 B). These results further suggest direct and/or indirect repression of \textit{agrA} by \textit{σ}^B. In the presence of the soil microbiota, environmental cues may trigger overexpression of \textit{agrA} in the line of the tight regulation of its activity according to the conditions of the habitat. Although non-significant, the levels of \textit{sigB} transcripts were higher in the \textit{ΔagrA} mutant in sterilised soil and no differences were recorded under biotic soil conditions. Altogether, these results are suggesting that interconnections between cell-cell communication and general stress response might be taking place. Altering either of these systems did affect fitness of \textit{L. monocytogenes} and inactivation of the two regulators resulted in an even stronger defect preventing the cells to cope with the biotic environment.

Overall, our results point to a model where \textit{σ}^B activity results in a repression of Agr transcription. This was observed in sterilised soil only. In biotic soil, sensing of environmental cues might trigger activation of the Agr system, resulting in increased transcription of the \textit{agr} operon, despite \textit{σ}^B activity. Taken together, these results suggest that AgrA and \textit{σ}^B global regulatory systems play important roles in the natural ecology of \textit{L. monocytogenes}.

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\section*{CONFLICTS OF INTEREST}

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
REFERENCES


CHAPTER 3

ACTIVATION OF AGR AND σ^B SYSTEMS DURING LISTERIA MONOCYTOGENES EGD-E BIOFILM DEVELOPMENT SUGGESTS REGULATORY OVERLAP

3.1. **PREAMBLE**

We have shown in the previous chapter that a synergistic activity between AgrA and $\sigma^B$ appears to contribute to the survival of *L. monocytogenes* in soil and roots colonisation, further suggesting a direct and/or indirect repression of agrA by $\sigma^B$, in the absence of the soil microbiota.

Here, we sought to investigate this interaction at the single cell level. As described in the introduction, both AgrA (Riedel *et al.*, 2009; Rieu *et al.*, 2007; Zetzmann *et al.*, 2019) and $\sigma^B$ (Lemon *et al.*, 2010; Raengpradub *et al.*, 2008; van der Veen and Abee, 2010b) activities have been extensively correlated with *L. monocytogenes* biofilm phenotype. The identification of an overlap between the Agr and the $\sigma^B$ regulons further suggests that these are central nodes in a complex regulatory network that governs the remodelling of the transcriptome during biofilm formation (Zetzmann *et al.*, 2019). In the food environment (Burgess *et al.*, 2016), as well as in the host’s gastrointestinal tract (Barbosa *et al.*, 2012), *L. monocytogenes* may encounter osmotic stress, the response to which also requires the $\sigma^B$ regulator (Abram *et al.*, 2008a), aside from influencing agrA transcription (Larsen and Jespersen, 2015).

Therefore, we aimed to study the role of the communication system Agr and the stress response factor $\sigma^B$ in *L. monocytogenes* biofilm development and osmotolerance, their activation over time, as well as the nature of the interconnection between these systems. For this, we resorted to a collection of fluorescent reporter systems on Agr- and $\sigma^B$-dependent promoters in the wild-type, $\Delta$agrA and $\Delta$sigB deletion mutants to assess the spatiotemporal expression of these systems during biofilm formation and estimate the changes in one regulator’s activity when the other is deactivated. The BioFlux microfluidic system was the instrument of choice to accomplish these objectives, as it not only kept a controlled environment (temperature and shear), it also permitted the visualisation of the biofilm formation and reporters expression over time.
3.2. Article

Original Research

Activation of Agr and σ^B systems during *Listeria monocytogenes* EGD-e biofilm development suggests regulatory overlap

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**ABSTRACT**

*Listeria monocytogenes* is a major concern in the food-production industry due to its ability to form resilient biofilms that are difficult to eliminate, which can increase the risk of food contamination and ultimately infection of the consumer. Either in food-production facilities or in the gastrointestinal tract of the host, this pathogen is exposed to osmotic stress and hydrodynamic shear. Two transcriptional regulatory systems of *L. monocytogenes*, the cell communication regulatory system Agr and the regulator of the general stress response $\sigma^B$ have been reported as central nodes in a complex regulatory network that governs the remodelling of the transcriptome in response to environmental changes. This study investigated the contribution of these systems when sessile *L. monocytogenes* faces osmotic stress. Biofilms of the parental *L. monocytogenes* EGD-e and two isogenic mutants lacking either *agrA* or *sigB* were quantified in medium with and without salt supplementation. To monitor the spatiotemporal activation of Agr and $\sigma^B$ regulons in the biofilm cells, fluorescent reporter fusions for these systems were obtained. These reporter systems fused an Agr-dependent promoter or a $\sigma^B$-dependent promoter with a green fluorescent protein gene. The reporters were then integrated into the genomes of the parental strain as well as two mutant derivatives. The resulting strains were used to study Agr and $\sigma^B$ activation during biofilm formation. Deletion of *agrA* or *sigB* resulted in increased static biofilm formation. Similarly, deletion of *agrA* modified sessile biofilm formation in a microfluidic flow chamber, while a reduction of biofilm production was observed after deletion of *sigB*. The Agr regulon is activated in an earlier stage of biofilm formation than $\sigma^B$, while the inactivation of one of these transcription regulators impacts the activation of the other. Temporal activation of Agr- and $\sigma^B$-dependent promoters was altered in the $\Delta$*sigB* and $\Delta$*agrA* mutants. Agr activation decreased in $\Delta$*sigB* background, while an earlier activation of $\sigma^B$ was observed in the $\Delta$*agrA* mutant than in the wild-type. However, when biofilm formed in hyperosmotic conditions, no activation of Agr was shown in the $\Delta$*sigB* background, while $\sigma^B$ was activated at later stages of biofilm development in the $\Delta$*agrA* mutant. This study suggests the involvement of Agr and $\sigma^B$ in biofilm formation and further highlights a link between Agr and $\sigma^B$ regulons during biofilm production.
**KEYWORDS**

Biofilm, microfluidic flow system, osmotic stress, fluorescent reporter system, promoter activity, overlap, bioflux, σ^B, Agr

**HIGHLIGHTS**

- Deletion of *agrA* or *sigB* resulted in changes in biofilm formation.
- *Agr* is activated at an earlier stage of biofilm development than σ^B.
- Temporal activation of *Agr* - and σ^B-dependent promoters was altered in the mutants.
- Hyperosmotic conditions cause a reverse chronological order of activation.

**INTRODUCTION**

*Listeria monocytogenes* is a facultative intracellular pathogen that has the capacity to transition from a saprophytic lifestyle to a highly virulent state within the mammalian host (Gray et al., 2006; Freitag et al., 2009). This foodborne pathogen is able to form resilient biofilms within a wide range of temperatures (4 to 42°C), and higher amounts of biofilm are formed at the optimal growth temperature 37°C than at 4°C (Di Bonaventura et al., 2008; Abdallah et al., 2014; Doijad et al., 2015; Tomićić et al., 2016). Generally, the composition of the growth medium has a significant impact on *L. monocytogenes* biofilm formation, but only a minimal effect results from changes in the growth temperature (Moltz, Martin, 2005).

Most studies on the establishment of *L. monocytogenes* biofilm were generated either statically (Sela et al., 2006; Pilchová et al., 2014), or in flow-cell systems for simulating dynamic conditions (Rieu et al., 2008; Harmsen et al., 2010; van der Veen, Abee, 2010). Biofilm formation increases survival, even when bacteria encounter hydrodynamic conditions (e.g., drains, pipes and within the host gastrointestinal tract). To characterize the effect of flowing liquid one usually refers to shear stress, which is the tangential force exerted per unit area by a fluid moving near a stationary wall (expressed in dynes/cm^2). The microfluidic
system BioFlux has been used to study biofilms of some species like \textit{Pseudomonas aeruginosa} and \textit{Staphylococcus aureus} (Diaz De Rienzo et al., 2016). It enables the assessment of biofilm formation under microfluidic growth conditions (Meyer et al., 2011), while accurately controlling temperature and fluid flow, plus permitting simultaneous growth of multiple biofilms with a large range of precisely controlled flow rates (Benoit et al., 2010). Recently, the BioFlux microfluidic system has been used to study \textit{L. monocytogenes} biofilm (Cherifi et al., 2017).

Due to its ability to cope with food-related conditions such as refrigeration temperatures, and high salt content, \textit{L. monocytogenes} can persist for long periods of time in food-processing facilities, especially when protected within biofilms (Doyle et al., 2001; Gardan et al., 2003; Tasara, Stephan, 2006; Ferreira et al., 2014; Zoz et al., 2017; Bucur et al., 2018; Rodriguez-Lopez et al., 2018). Despite being an efficient tool for reducing \textit{L. monocytogenes} from surfaces in food processing facilities (Sheen et al., 2010; Soyer, Dumenil, 2011; Giao, Keevil, 2013), shear stresses do not fully eradicate resilient biofilms in food-processing reservoirs such as floors, drains and pipes (Di Ciccio et al., 2012; Liu et al., 2016). In the human body, \textit{L. monocytogenes} encounters as well several shear forces, from the mouth to the gut, on the surface of epithelial cells or in the blood circulation during advanced stages of infection. These form barriers to pathogen colonization and invasion of host cells (Davies, 1995; Soyer, Dumenil, 2011; Hansson, 2012). The intensities of these shear stresses vary widely according to the vessel types, size, organ and location (0-100 dynes/cm\textsuperscript{2}) (Soyer, Dumenil, 2011). Consequently, pathogens have evolved sophisticated molecular strategies to overcome the shear forces, such as membrane anchored complexes that enable controlled deceleration, tight adhesion to surfaces, and penetration through the host cell membrane (Carruthers, Suzuki, 2007; Nauman et al., 2007; Harvey et al., 2012; Moriarty et al., 2012; Harker et al., 2014).

Biofilm formation by \textit{L. monocytogenes} can be viewed as five distinct steps: attachment, cell-cell adhesion, proliferation, maturation and dispersion (Colagiorgi et al., 2017). Two transcriptional regulatory systems of \textit{L. monocytogenes}, Agr and \(\sigma^8\) have been reported as central nodes in a complex regulatory network that governs the remodelling of the transcriptome during biofilm formation (Zetzmann et al., 2019). The loss of each regulator resulted in changes at different stages of \textit{L. monocytogenes} biofilm development; reduced
adherence on early biofilm formation on static abiotic surfaces in the ΔagrA mutant (Rieu et al., 2007), while the ΔsigB mutant showed a significant decrease in the amount of biofilm formed (van der Veen, Abbe, 2010). However, insights into the temporal activation of these regulators during biofilm development could help to further elucidate their contribution to this process.

Osmotic stress in food mostly arises as a result of increased concentrations of salts or sugars that are added to improve the sensory properties and as preserving agents to increase the shelf life (Burgess et al., 2016). Moreover, osmotic stress is also one of the adverse conditions that *L. monocytogenes* encounters in the host gastrointestinal tract (Louis, O’Byrne, 2010; Barbosa et al., 2012). In response to elevated concentrations of salt, *L. monocytogenes* accumulates osmolytes such as carnitine and glycine betaine in the cytoplasm to reduce osmotic pressure and water loss (Duche et al., 2002), while stabilizing the structure of enzymes and their function during stress (Lippert, Galinski, 1992). In *L. monocytogenes*, salt tolerance also requires the activity of σB (Fraser et al., 2003; Abram et al., 2008). However, to our knowledge, studies reporting the involvement of Agr in response to osmotic stress are scarce. Larsen and Jaspersen (2015) have shown that hypo-osmotic stress promoted a decrease in the transcription levels of agrA, which suggested that this transcription regulator might have a role in salt tolerance (Larsen, Jespersen, 2015).

In this work, both flow and static methods were used to investigate the contribution of the Agr and σB systems when sessile *L. monocytogenes* faces osmotic stress. Employing eGFP reporter systems with Agr- and σB-dependent promoters, the spatiotemporal activation of these systems during biofilm formation was determined.
**MATERIALS AND METHODS**

**Strain and culture conditions and inoculum preparation**

In this study, *Listeria monocytogenes* EGD-e was used as the parental strain (Glaser et al., 2001). The isogenic mutant derivatives ΔagrA (Rieu et al., 2007) and ΔsigB (Marinho et al., 2019) were constructed in previous work. A collection of reporter systems for measuring Agr and σ^B_8 activity was constructed as previously described (Utratna et al., 2012). A reporter fusion of the Agr-dependent promoter from agrB (located 690 pb upstream of the start codon) fused with the green fluorescent protein gene *gfp* was previously constructed in pGID128 (Rieu et al., 2008). The reporter plasmid pGID310 was derived from pGID128 with the fusion of the bgaB gene of *Bacillus stearothermophilus* (Gerth et al., 1998) downstream of the P_{agr}::*gfp* construct to enable this vector with an alternative P_{agr-}-reporter system. The reporter plasmid pKSV7-P_{lmo2230}::*egfp* was designed to contain the fusion of the enhanced green fluorescent protein (*egfp*) cloned downstream the highly σ^B_8-dependent promoter region of *lmo2230* (443 bp sequence upstream of the start codon), described in detail by Utratna et al. (2012). These plasmids were transformed into *L. monocytogenes* EGD-e and the ΔagrA and ΔsigB derivative mutants, and integrated into the chromosome as previously described (Utratna et al., 2012). All primers, plasmids and strains used in this study are listed in Table 3.1. Cultures of all *L. monocytogenes* strains (Table 3.1) were visualised under the microscope for fluorescence due to Agr- and σ^B_8-promoter-driven green-fluorescent protein gene expression according to the method of Utratna et al. (2012). *L. monocytogenes* strains were grown in tryptic soy broth (TSB) or TSB agar (Conda) at 37°C unless otherwise stated. Cells were grown with continuous shaking from a starting OD_{600} = 0.05 for 16 h (stationary phase). The volume of the medium occupied no more than 10% of the flask volume to ensure sufficient aeration. *Escherichia coli* strains used as intermediate vector hosts were grown in brain heart infusion (BHI) broth or BHI agar (LabM) at 30°C. When appropriate, cultures were supplemented with antibiotics as follows: chloramphenicol (Cml) 10 μg/ml for *L. monocytogenes*; ampicillin (Amp) 100 μg/ml or erythromycin (Erm) 250 μg/ml for *E. coli*. 

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Table 3.1. Strains, plasmids and primers used in this study.

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<th>Name</th>
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<th>Further information</th>
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<td>Primers</td>
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<td>CAGGAAACAGCTATGAC</td>
<td>Forward primer for flanking insert in the pKSV7 vector.</td>
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<td>M13_R</td>
<td>GTAAACGACGGCCAG</td>
<td>Reverse primer for flanking insert in the pKSV7 vector.</td>
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<td>Rieu et al., 2008</td>
<td>Derivative of pGF-EM carrying the P_{agr::gfp} fusion.</td>
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<tr>
<td>pKSV7</td>
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<td>pUC18 and pBD95 integrated shuttle vector carrying a temperature sensitive oriC from pE194.</td>
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<tr>
<td>pGID310</td>
<td>This study</td>
<td>Reporter plasmid derivative of pGID128 carrying the P_{agr::gfp::bgaB} fusion.</td>
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<td>Utratna et al., 2012</td>
<td>Reporter plasmid derivative of pKSV7 carrying the P_{lmo2230::egfp} fusion.</td>
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<tr>
<td>Strains</td>
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<tr>
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<td>Source</td>
<td>Further information</td>
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Static biofilm formation

Biofilm assays in microtiter plates were adapted from a method previously described by Djordjevic et al. (2002). One millilitre of an overnight culture was centrifuged at 13,000 g for 2 min. The pellet was washed once in 1 ml PBS, centrifuged and resuspended in 1 ml PBS. Five microliters of the washed cells were added to 5 ml of either TSB broth or TSB supplemented with 0.5 M NaCl (TSBN). Two hundred microliters of this resuspension were transferred to a flat bottomed 96-well tissue culture plate (Sarstedt) with six technical replicates for each strain/biological replicate. Sterile media was added to each plate as a control. The wells surrounding the samples were filled with sterile media or PBS to help prevent evaporation from sample wells. The plate was subsequently incubated statically for 48 h, unless otherwise specified, at the required temperature. After incubation, the media was carefully removed from all wells using a pipette and each well was washed 3 times with 200 μl PBS. The plate was allowed to dry at 40-45°C for 45 min and 150 μl of a 1% (w/v) crystal violet (CV) (Sigma-Aldrich) solution (10 g crystal violet in 1000 ml dH2O) was then added to each well. The plate was incubated statically at 37°C for 30 min and excess crystal violet was removed. The plate was washed twice with 200 μl PBS and then gently rinsed with dH2O to remove excess crystal violet. Finally, 160 μl 95% ethanol was added to each well. The plate was incubated for a further 30 min at room-temperature with gentle agitation, or until the crystal violet dissolved. The OD\textsubscript{595} value for each well was recorded using a Tecan Sunrise plate reader.

BioFlux assay of biofilm formation

The BioFlux Z1000 system with 24-well plates (Fluxion biosciences, South San Francisco, California, USA) was used for biofilm formation. The protocol developed by Benoit et al. (2010) and Tremblay et al. (2015) for other species was adapted for \textit{L. monocytogenes} biofilm formation. Overnight cultures (\textit{OD}\textsubscript{600} = 2.0) of \textit{L. monocytogenes} strains were centrifuged and resuspended in ten times the initial volume of fresh pre-warmed TSB or TSBN medium. One hundred microliters of these cultures were added to the output well of the BioFlux instrument then injected at 0.5 dyn/cm\textsuperscript{2} (0.05 Pa) of shear stress for 30 s into the microfluidic flow channels that had been wetted with a fresh pre-warmed TSB medium. Plates were incubated
at 37°C for 2 h without flow to allow bacteria attachment to the surface of the chambers, and the wells were equilibrated by adding 100 μl of pre-warmed sterile medium in the input wells. After 2 h, the input wells were filled with 1 ml of pre-warmed TSB or TSBN and the 24-well plates were incubated at 37°C with shear stress. The shear stress applied was of 0.3 dyn/cm² (0.03 Pa) for 24 h and the flow was run at 30 μl/h. Biofilm growth was monitored by taking pictures every 10 min for 24 h in either brightfield and FITC fluorescence using an inverted fluorescence microscope (Axio Observer Z1, Zeiss, Germany) equipped with a 10X objective and a digital camera (Retiga EX; Q Imaging).

Quantification of acquired BioFlux biofilm images

The method used for biofilm quantification was adapted from Moormeier et al. (2014). Using BioFlux Montage software (Fluxion Biosciences, Inc.), representative brightfield and fluorescence images were processed and their brightness adjusted. For brightfield images, a threshold was set using the Threshold tool and Slider to include all dark objects (biofilm cells) within each image. The total area covered by dark objects and expressed as a percentage was designated as the percentage of biofilm coverage. This figure was recorded and plotted over time. For fluorescence images, a threshold similar to that described above was set to cover all light objects (fluorescent cells) in each image. It was designated as percentage of fluorescence coverage. All data were analysed and plotted over time using GraphPad Prism version 7.0a for Mac OS X (GraphPad Software, La Jolla, CA).

Statistical analysis

The significance of the differences in biofilm amounts was assessed by paired t-test using GraphPad Prism version 7.0a for Mac OS X (GraphPad Software, La Jolla, CA). Data of at least two independent experiments were analysed, each containing at least two technical replicates.
RESULTS

Deletion of *agrA* and *sigB* alters biofilm under osmotic stress conditions

*L. monocytogenes* biofilms were incubated without shaking for 48 h at 37°C in TSB and TSB supplemented with 0.5 M NaCl (TSBN). Measurements of bound crystal violet (CV) showed that all strains formed lower amounts of biofilm in TSBN than in TSB (Fig. 3.1). Amounts of biofilm formed by the Δ*sigB* mutant remained unchanged between media, while significantly smaller amounts of biofilms were formed by wild-type and Δ*agrA* strains in both conditions. In comparison to the parental strain, although the deletion of *agrA* did not affect biofilm production in TSB, the Δ*agrA* mutant showed a small but significant increase in biofilm formation under hyperosmotic conditions (Fig. 3.1). Additionally, the Δ*sigB* mutant showed significantly higher biofilm production than the wild-type under both growing conditions. To determine if these phenotypes would also be observed under other growth conditions, biofilm formation was assessed under dynamic conditions.

![Graph](image_url)

**Figure 3.1.** Static biofilm formation of *L. monocytogenes* EGD-e (WT) and its isogenic *agrA* (Δ*agrA*) and *sigB* (Δ*sigB*) deletion mutants after 48 h at 37°C in rich medium TSB and TSB supplemented with 0.5 M NaCl (TSBN). OD₅₉₅ measurements of CV-stained biofilms. The data represents the mean from three independent experiments, each containing three technical replicates. Error bars represent
standard deviation. Asterisks represent \( p \)-values (ns = not significant, * \( p \)-value <0.05, ** \( p \)-value <0.001, *** \( p \)-value <0.0001) calculated using a paired \( t \)-test to determine statistical differences between the wild-type and the mutant strains (on top of each column) or between media.

When \( L.\ monocyto
gen\)es EGD-e was grown at 37\(^\circ\)C in TSB under dynamic conditions in a BioFlux microfluidic flow system, after the initial attachment of cells (0 h), a gradual increase on biofilm coverage was observed over time (Fig. 3.2 A). After 12 h incubation, dispersed biofilm colonies were observed throughout the channel. Lower initial attachment was observed with the \( \Delta agrA \) mutant but 24 h biofilms were similar to the wild type. The \( \Delta sigB \) mutant showed lower biofilm production, but the biofilm was more uniformly distributed and some filaments and microcolonies were observed by the end of the 24 h incubation in rich medium TSB (Fig. 3.2 A). Despite showing a quicker multiplication within the first four hours of the experiment, the \( \Delta sigB \) mutant ended up producing about half of the biofilm coverage of the WT (~40% biofilm coverage) after 24 h (Fig. 3.2 A).

In contrast, when subjected to NaCl supplemented medium (TSBN), all strains produced less biofilm with scattered elongated chain structures (Fig. 3.2 B). Under this condition, minute amounts of sessile \( L.\ monocyto
gen\)es EGD-e were observed and significantly less biofilm was produced in comparison to the medium without NaCl supplementation. The amounts of biofilms formed by the mutant strains were lower in TSBN than in TSB (Fig. 3.2 B). The \( \Delta agrA \) mutant produced more biofilm than all the other strains. Conversely, lower attachment was recorded with the \( \Delta sigB \) mutant, eventually reaching similar biofilm coverage as the WT after 17 hours and even slightly overcoming it (~7% biofilm coverage) (Fig. 3.2 B).
Figure 3.2. Biofilm formation of *L. monocytogenes* EGD-e (WT) and isogenic ΔagrA and ΔsigB deletion mutants over a 24 h period at 37°C under flow. (A) Biofilm formed in rich medium TSB. (B) Biofilm formed in rich medium TSB supplemented with 0.5 M NaCl (TSBN). Pictures were taken followed to cells attachment (0 h), after 6 h, 12 h and 24 h and are representative of two independent experiments, each containing three technical replicates. Plots of the biofilm growth as the mean percentage of biofilm coverage in 2 h intervals over 24 h of growth in TSB or TSBN. The data represent
the means of two independent experiments, each containing at least two technical replicates. Error bars were omitted for clarity.

Figure 3.2 (continued).
Temporal activation of Agr and σ^B-dependent promoters during sessile growth

Comparison of biofilm produced by *L. monocytogenes* EGD-e parental strain, and the ΔagrA and ΔsigB knockout mutants under static and dynamic conditions suggested that these regulators contributed to biofilm development in rich medium, albeit in different ways depending on the sessile growth conditions. To further investigate when the Agr and σ^B systems were activated in the course of sessile growth, reporter fusions constructed in the wild type and mutant backgrounds were analysed. The *lmo2230* gene, which encodes a putative arsenate reductase, is preceded by a strong σ^B-dependent promoter, which was shown before to generate strongly σ^B-dependent fluorescence when fused with the enhanced green fluorescent protein gene (*egfp*), making this a reliable indicator of σ^B activity in *L. monocytogenes*, particularly under osmotic stress (Utratna et al., 2012). Similarly, the agr promoter region (located 690 bp upstream of the start codon of *agrB*) was previously fused with the *gfp* gene and shown to accurately report agr activity (Rieu et al., 2007). Activity of the *P_{agr}::*gfp and *P_{lmo2230}::*egfp reporter fusions was confirmed in the wild-type (DG311E and CM015E), ΔagrA (CM011E) and ΔsigB (CM12E) reporter strains by visualization of fluorescent stationary phase cells through fluorescent microscopy. As expected, no fluorescence was observed on the Agr (CM14E) and σ^B (CM013E), reporter strains in the absence of agrA and sigB, respectively. Therefore, reporter strains containing *P_{agr}::*gfp and *P_{lmo2230}::*egfp promoter fusion plasmids integrated into the genome were used to study the spatiotemporal activation of Agr and σ^B systems during biofilm formation in dynamic conditions in TSB at 37 °C. Brightfield and epifluorescence images representative of biofilms formed at the beginning of the experiment (0 h), and after 6-, 12- and 24-h were recorded for each strain (Fig. 3). Activity of the of *agr* and *lmo2230* promoters was detected in wild type biofilms throughout the incubation period. In the WT, Agr activity raised after 10 h and was maximal at 16 h of biofilm growth (>10% Fluorescence Coverage), from which point Agr activity steadily declined, while biofilm development reached a steady state (~20% Biofilm Coverage) (Fig. 3.3 A). Similarly, *lmo2230* activation was detected after 10 h and σ^B activity increased until the end of the experiment after 24 h incubation (>15% Fluorescence Coverage) (Fig. 3.3 B). Deletion of sigB negatively affected activity of Agr and fluorescence was not detected before 10 h incubation from which point its activity was proportional to biofilm formation (Fig. 3.3 C). The maximum of Agr activity was recorded after 24 h (~7% Fluorescence Coverage) (Fig. 3.3 C). When the
Agr system was not functional, σ^B activity was not detected within the first 4 hours of incubation. It then increased and reached a maximum after 16 h of incubation (~12% Fluorescence Coverage) before decreasing steadily until the end of the experiment (Fig. 3.3 D). Fluorescence was observed only in subpopulations throughout biofilm development in rich medium. Overall, the whole population did not show the same behaviour, as Agr and σ^B activity was observed in microcolonies throughout the biofilms. These results indicated a temporal activation of Agr- and σ^B-dependent promoters during biofilm development in rich medium under flow, as well as a possible interconnection of the Agr and σ^B systems, as variations in the activities of these promoters resulted from the absence of the other regulatory system.
Figure 3.3. Biofilm formation of *L. monocytogenes* reporter strains over a 24 h period at 37°C under flow in rich medium TSB. *L. monocytogenes* EGD-e reporter strains for (A) Agr (WT, *P_{agr}::gfp*), (B) σ^B (WT, *P_{mo2230}::egfp*), (C) Agr in the ΔsigB mutant background (ΔsigB, *P_{agr}::gfp*), and (D) σ^B in the ΔagrA mutant background (ΔagrA, *P_{mo2230}::egfp*). Brightfield and fluorescent pictures were taken followed to cells attachment (0 h), after 6 h, 12 h and 24 h and are representative of two independent experiments, each containing three technical replicates. The plots depict biofilm growth as the mean
percentage of biofilm coverage and the mean fluorescence coverage in 2 h intervals over 24 h of growth in TSB. The data represent the means from two independent experiments, each containing at least two technical replicates. Error bars were omitted for clarity.

Figure 3.3. (continued).
When the medium was supplemented with NaCl, activation of Agr (Fig. 3.4 A) and σ^B (Fig. 3.4 B) was detected. Throughout the experiment, Agr activity was lower than 1% Fluorescence Coverage, and a maximum was observed after 22 h (Fig. 3.4 A). σ^B activity was below 1.7% Fluorescence Coverage over the experiment, and it peaked after about 14 h (Fig. 3.4 B). Under osmotic stress, Agr activity in the ΔsigB mutant was barely detectable during biofilm development (Fig. 3.4 C). Although in the ΔagrA background lmo2230 promoter activity was initially low (<0.5% Fluorescence Coverage), it increased after 15 h reaching a maximum of over 1.5% Fluorescence coverage after 19 h, followed by a slight decrease by the last hours of the experiment (Fig. 3.4 D). Data suggested that when under osmotic stress, L. monocytogenes EGD-e activates the σ^B system at the early stages of biofilm development, whereas Agr activation is delayed. However, Agr activity was almost undetectable in the absence of σ^B, while the inactivation of the Agr system resulted in a delayed activation of σ^B.
Figure 3.4. Biofilm formation of *L. monocytogenes* reporter strains over a 24 h period at 37°C in flowing conditions under osmotic stress in rich medium TSB supplemented with 0.5 M NaCl. *L. monocytogenes* EGD-e reporter strains for (A) Agr (WT, P_{agr::gfp}), (B) σ^B (WT, P_{mo2230::egfp}), (C) Agr in the ΔsigB mutant background (ΔsigB, P_{agr::gfp}), and (D) σ^B in the ΔagrA mutant background (ΔagrA, P_{mo2230::egfp}). Brightfield and fluorescent pictures were taken followed to cells attachment (0 h), after 6 h, 12 h and 24 h and are representative of two independent experiments, each containing three
technical replicates. The plots depict biofilm growth as the mean percentage of biofilm coverage and the mean fluorescence coverage in 2 h intervals over 24 h of growth in TSBN. The data represent the means from two independent experiments, each containing at least two technical replicates. Error bars were omitted for clarity.

Figure 3.4 (continued).
**DISCUSSION**

The foodborne bacterium *L. monocytogenes* is a facultative intracellular pathogen that can use different routes of transmission to move between the outdoors, food, and the host environments (NicAogáin, O'Byrne, 2016). When organised as structured biofilms, *L. monocytogenes* increases resistance to numerous stress conditions it encounters and this enhances its persistence in diverse habitats (Pan et al., 2006; van der Veen, Abbe, 2011). The structure of microbial biofilms varies in response to hydrodynamic conditions (Stickler, 1999; Wimpenny et al., 2000; Rieu et al., 2008). The structure of *L. monocytogenes* biofilm under static conditions has been intensively studied, although the literature contains a variety of different views on how the biofilm is organised. While some described the organisation as a honeycomb-like morphology (Marsh et al., 2003; Guilbaud et al., 2015), others reported the development of unorganized biofilms (Chavant et al., 2002; Borucki et al., 2003; Chavant et al., 2004; Rieu et al., 2008). Less information is available about *L. monocytogenes* biofilm structure under hydrodynamic conditions, which are typically more relevant to food processing environments where biofilm can often be problematic. Despite providing a constant source of nutrients widely distributed across the biofilm, the shear stress generated by fluid flow reduces adhesion of bacterial communities to surfaces, promoting distinct colonization patterns with consequences for biofilm growth (Thomen et al., 2017). Significantly greater biofilm volume and thickness was formed by *L. monocytogenes* under dynamic conditions than under static conditions and multilayer cell structures formed a network of knitted chains that could structure microcolonies (Rieu et al., 2008). Others used dynamic conditions in the Bioflux system to investigate nutrient deprivation on the structure of biofilms (Cherifi et al., 2017). Our results establish that, under dynamic conditions in the growth media we studied, *L. monocytogenes* produces uniformly dispersed biofilm, with some ball-shaped structures (Fig. 3.2 A).

A multitude of parameters such as nutrient availability, temperature, the nature of colonised surface and the presence of other microorganisms influences biofilm formation by *L. monocytogenes* (Moltz, Martin, 2005; Di Bonaventura et al., 2008; Tomičić et al., 2016; Cherifi et al., 2017). This study demonstrated that the presence of osmotic stress also influences *L. monocytogenes* biofilm formation. Indeed, less biofilm was produced under osmotic stress
conditions either under static (Fig. 3.1 and 3.2 B) or under dynamic conditions, where scattered elongated chain structures were observed (Fig. 3.2 B). It is believed that this particular morphology is a consequence of shear stress as it was not observed in static biofilm (Cherifi et al., 2017). The production of filamentous structures when L. monocytogenes is exposed to a range of stress conditions such as high osmolarity, has been described as an adaptive mechanism to increase its survival under harsh conditions (Bereksi et al., 2002). However, these long chain structures might as well result from the flow causing new cells to attach downstream from the parent cell.

As the initial step of biofilm development, cell attachment to surfaces is critical for the formation of a bacterial biofilm (Palmer et al., 2007; Colagiorgi et al., 2017). Our results showed that the Agr system is involved in this first step of biofilm formation (Fig. 3.2 A) as described previously (Rieu et al., 2007; Riedel et al., 2009; Zetzmann et al., 2019) and highlight its role in the regulation of L. monocytogenes biofilm development. However, our data showed that mature static biofilm production in rich medium (Fig. 3.1) was not affected by the inactivation of the Agr communication system. Previous data reported that L. monocytogenes biofilm development was increased by the deletion of agrA within the initial 24 h, but no more differences were recorded at later stages of biofilm formation (Garmyn et al., 2012). Moreover, the cell communication system Agr does not seem to play any role in biofilm architecture, as no alterations of biofilm morphology were caused by the deletion of agrA (Fig. 3.2 A), although the activity of Agr progressively increased after attachment and was proportional to biofilm formation until stationary growth phase (Fig. 3.3 A). Heterogeneous activity of the agr system across L. monocytogenes biofilm oscillating over time was also previously observed (Rieu et al., 2007). Altogether, data suggests that Agr-dependent regulation may be transitory; following adhesion, L. monocytogenes undergoes profound gene expression alterations during sessile growth, as has been proposed for other bacteria. The activation of Agr in subpopulations across the biofilm fits the bet-hedging theory; the generation of offspring with different phenotypes could facilitate survival of clonal populations to ever-changing environmental conditions (Veening et al., 2008a; Veening et al., 2008b).
Deletion of agrA resulted in higher biofilm production under osmotic stress than the wild-type (Fig. 3.1 and 3.2 B), implying that the Agr communication system is involved in the response of L. monocytogenes to osmotic stress during biofilm development. Interestingly in BHI broth, an in-frame deletion of agrA did not affect the growth in the presence of 9% NaCl during incubation at various temperatures (Williams et al., 2005) and a decreased transcription of agrA in L. monocytogenes was promoted after incubation in low rather than high NaCl content cheese (Larsen, Jespersen, 2015). In contrast to what might be expected under these conditions, Agr activity was residual in the WT strain throughout biofilm formation in hyperosmotic conditions (Fig. 3.4 A).

The role of σB in L. monocytogenes biofilm formation remains mostly unknown. Here we showed that the deletion of the sigB gene increases the production of static biofilm in rich medium, supplemented with NaCl or not, demonstrating a regulatory role for σB in the development of biofilms under these conditions (Fig. 3.1). This is in contrast to a previous report showing that inactivation of σB resulted in a significant reduction of both static and continuous-flow biofilms (van der Veen, Abee, 2010). The differences in culture conditions such as temperature, medium and methods used in these studies could be the reason for the discrepancies observed, as suggested previously (Combrouse et al., 2013; Cherifi et al., 2017). However, a relative increase in biofilm production by the ΔsigB strain was also reported by O’Donoghue (2017), but only in chemically defined medium at 37°C, further indicating that differences in growth methods might result in different phenotypes (O’Donoghue, 2017). Our data suggest that inactivation of σB impacts susceptibility to shear stresses as less biofilm was formed in dynamic conditions by the sigB deletion mutant (Fig. 3.2). Particularly, the absence of σB hampered cell attachment to the surface and consequently biofilm development under osmotic stress in dynamic conditions. Heterogeneous σB activation when L. monocytogenes develops as biofilm under flow was also observed (Fig. 3.3 B and 3.4 B), further supporting the bet-hedging strategy and that not all cells may perceive osmotic stress in the same way (Utratna et al., 2012). This phenotype has been investigated in other bacteria and partly explained by fluctuations in the amount of cellular components together with the noise and the asynchrony in gene expression (Elowitz et al., 2002; Guldimann et al., 2017). Alternatively, cells within the biofilm could be in different metabolic states due to subtle differences in their local microenvironments (cell density, nutrient diffusion, pH, etc.). Persistent cells are
dormant, metabolically inactive bacterial cells that present an increased ability to survive environmental stresses (Lewis, 2010; Wen et al., 2011; Knudsen et al., 2013; Wood et al., 2013). The presence of subpopulations comprised by persister cell in bacterial biofilms has been observed (Singh et al., 2009; Wood, 2017) and poses as a plausible motive for the observed heterogeneous activation of these transcription regulators across the biofilms.

The $\sigma^B$ activity was low following cell attachment, and increased thereafter (Fig. 3.3 B). The stress response factor $\sigma^B$ is strongly active in stationary phase cells or in cultures supplemented with NaCl (Utratna et al., 2011). In *L. monocytogenes*, $\sigma^B$ has been shown to negatively affect growth rate in response to osmotic stress, as shown by an increase in the growth rate of a mutant lacking *sigB* under conditions of mild osmotic stress (Abram et al., 2008). In biofilm developing under osmotic stress in dynamic conditions, the activity of $\sigma^B$ was generally low (Fig. 3.4 B).

The hypothesis of an overlap in *L. monocytogenes* between cell-cell communication (Agr) and the general stress response ($\sigma^B$), is supported by the differential expression of a remarkably large number of genes connected to stress response and resistance to atypical conditions when the Agr communication system is inactivated (Garmyn et al., 2012; Zetzmann et al., 2019). Together with the numerous studies reporting the involvement of Agr or $\sigma^B$ in biofilm development (Rieu et al., 2007; Rieu et al., 2008; van der Veen, Abee, 2010) this led us to investigate the combined roles of these two regulons in the context of dynamic biofilm development. Indeed, the deletion of *agrA* and *sigB* altered $\sigma^B$ and Agr activity, respectively (Fig. 3.3). Results suggest a lower activity of Agr in the absence of $\sigma^B$ (Fig. 3.3 C). In the absence of $\sigma^B$ (Fig. 3.3 C), Agr activity is kept more or less constant throughout biofilm formation, unlike what was observed in the wild-type (Fig. 3.3 A). Although there is little Agr activity in the wild-type cells of biofilms grown under osmotic stress (Fig. 3.4 A), the deletion of *sigB* led to a further decrease in Agr activity (Fig. 3.4 C). Conversely, activation of $\sigma^B$ in biofilm is lower in the absence of the communication system Agr in rich medium with (Fig. 3.4 B and D) or without NaCl supplementation (Fig. 3.3 B and D). Overall, these observations support the view that there is crosstalk between the Agr and $\sigma^B$ regulons, as variations in the activity of these promoters resulted from the absence of the regulator of the other system during continuous flow-biofilm production. It is perhaps not surprising that a system
dedicated to sensing cell density should be influenced by a regulatory system whose primary role appears to be optimising survival under stressful conditions, and vice versa. The transition to a sessile lifestyle brings with it new challenges in relation to space, competition and nutrient availability but also produces a strong protective state that contributes to the long-term persistence of *L. monocytogenes* in the many different environments it inhabits.

In conclusion, this work has shed some light on the role that Agr and σ^B systems play in *L. monocytogenes* EGD-e during biofilm formation and under hyperosmotic conditions. The Agr regulon is activated during the phase of growth of the biofilm, while the activation of the σ^B regulon occurs later on during late stationary phase. The inactivation of one of these transcription regulators impacts the activation of the other; Agr activity decreases in the absence of sigB, while activation of σ^B occurs earlier in the process of biofilm development in the absence of AgrA. However, in biofilm formed under hyperosmotic conditions, σ^B is activated in a primary stage of biofilm development while Agr activation is delayed. Moreover, the presence of salt in the medium results in no activation of Agr when sigB is deleted, while σ^B activation is postponed in the absence of AgrA. This study suggests the involvement of these two transcriptional regulators in biofilm formation and further highlights a link between Agr and σ^B regulons during biofilm production under dynamic conditions. Nonetheless, the Agr and σ^B co-regulated genes that contribute to biofilm formation under different environmental conditions remain to be elucidated in future studies.

**AUTHOR CONTRIBUTION**

CMM, PP, and COB: conceived and designed the experiments. CMM and DG: performed the experiments. CMM, LG, PP and COB: analysed the data. CM and COB: wrote the paper. All authors read and approved the final manuscript.

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CHAPTER 4

THE $\sigma^B$-DEPENDENT REGULATORY sRNA Rli47

REPRESSIONS Isoleucine Biosynthesis in Listeria

Monocytogenes Through a Direct Interaction

With the IlvA Transcript

4.1. PREAMBLE

The literature review has allowed for a synthesis of the main information on the AgrA and \( \sigma^B \) transcription regulators on *L. monocytogenes*. Moreover, in the chapters 2 and 3 we have suggested a synergistic effect between these two systems and a direct and/or indirect repression of *agrA* by \( \sigma^B \) when mimicking the telluric environment, as well as observed changes in the activation of one regulator in the absence of the other during biofilm formation and in the presence of osmotic stress. However, the molecular mechanisms responsible for this crosstalk remained occluded.

In *S. aureus*, these two systems are interconnected in a way that \( \sigma^B \) represses production of RNAIII (Bischoff *et al.*, 2001; Lauderdale *et al.*, 2009), a regulatory sRNA, which is the intracellular effector of the Agr system (Boisset *et al.*, 2007). Although an overlap between AgrA and \( \sigma^B \) regulons suggested a crosstalk between these two regulators in *L. monocytogenes* (Garmyn *et al.*, 2012), up to this date no putative orthologues of RNAIII have identified *in silico* in the *L. monocytogenes* genome. Despite being highly \( \sigma^B \)-dependent (Mujahid *et al.*, 2012; Oliver *et al.*, 2009; Toledo-Arana *et al.*, 2009), the sRNA Rli47 has also been described as part of the AgrA regulon (Vivant *et al.*, 2015), suggesting its transcription might be affected by both regulators. Thus, we hypothesized this sRNA could be mediating the interconnection of the two systems in *L. monocytogenes*. Several studies have been suggesting a biological role of Rli47 in the virulence process (Mraheil *et al.*, 2011; Toledo-Arana *et al.*, 2009) and in response to oxidative stress (Mujahid *et al.*, 2012). However, this remained undetermined, posing as another objective of this investigation.

In this study, we mapped Rli47 in the genome of *L. monocytogenes* EGD-e, and determined its true size and structure. Using an *in silico* approach, target mRNAs were predicted and further confirmed *in vitro* through binding assays. Phenotypic testing and transcriptomic approaches led to confirm the impact of Rli47 on the BCAA biosynthesis. Differential transcriptome analysis by RNA-seq was used to further identify other hypothetical targets of Rli47.
The $\sigma^B$-dependent regulatory sRNA Rli47 represses isoleucine biosynthesis in *Listeria monocytogenes* through a direct interaction with the *ilvA* transcript

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The $\sigma^B$-dependent regulatory sRNA Rli47 represses isoleucine biosynthesis in *Listeria monocytogenes* through a direct interaction with the *ilvA* transcript

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**ABSTRACT**

The facultative intracellular pathogen *Listeria monocytogenes* can persist and grow in a diverse range of environmental conditions, both outside and within its mammalian host. The alternative sigma factor Sigma B ($\sigma^B$) plays an important role in this adaptability and is critical for the transition into the host. While some of the functions of the $\sigma^B$ regulon in facilitating this transition are understood the role of $\sigma^B$-dependent small regulatory RNAs (sRNAs) remain poorly characterized. In this study, we focused on elucidating the function of Rli47, a $\sigma^B$-dependent sRNA that is highly induced in the intestine and in macrophages. Using a combination of *in silico* and *in vivo* approaches, a binding interaction was predicted with the Shine-Dalgarno region of the *ilvA* mRNA, which encodes threonine deaminase, an enzyme required for branched-chain amino acid biosynthesis. Both *ilvA* transcript levels and threonine deaminase activity were increased in a deletion mutant lacking the *rli47* gene. The *Δrli47* mutant displayed a shorter growth lag in isoleucine-depleted growth media relative to the wild-type, and a similar phenotype was also observed in a mutant lacking $\sigma^B$. The impact of the $\Delta rli47$ on the global transcription profile of the cell was investigated using RNA-sequencing and a significant role for Rli47 in modulating amino acid metabolism was uncovered. Taken together, the data point to a model where Rli47 is responsible for specifically repressing isoleucine biosynthesis as a way to restrict growth under harsh conditions, potentially contributing to the survival of *L. monocytogenes* in niches both outside and within the mammalian host.

**Key terms**

Listeria monocytogenes; Rli47; Sigma B; Isoleucine biosynthesis; sRNA; Threonine deaminase

**Introduction**

The Gram-positive bacterium *Listeria monocytogenes* is a facultative intracellular pathogen widely found in the environment [1]. It is capable of colonizing the mammalian host following ingestion by susceptible individuals. Following a successful transit through the gastrointestinal tract, it can pass through the epithelial layer and replicate intracellularly in several organs, causing the life-threatening infection listeriosis [2,3]. Although the number of cases is small, the high mortality rate amongst infected individuals (typically 20%) makes listeriosis a significant public health concern [4–6]. The stress-activated sigma factor $\sigma^B$ plays a pivotal role in allowing *L. monocytogenes* to modulate its transcriptional response to a variety of harsh environments both outside and within the host [7].

While transitioning from a saprophytic to a host-associated state, *L. monocytogenes* relies on sensing host-specific signals that affect the transcription regulators CodY and PrfA and triggers the initiation of its virulence program [8–12]. In addition to these important regulatory proteins, it is now clear that small regulatory RNAs (sRNAs), non-coding transcripts of about 50 to 500 nucleotides long, can contribute to the control of virulence gene expression at the post-transcriptional level [13,14]. The genome of *L. monocytogenes* includes about 300 small non-coding RNAs, of which 154 are proposed to be trans-acting sRNAs [15]. Trans-acting sRNAs are located distally from the genes encoding their target RNAs and typically have only limited complementarity. The transcription of six sRNAs (SbrA, Rli47, Rli33-1, Anti-LhrC-5, Anti2270 and Rli95) of *L. monocytogenes* depends on the stress-inducible sigma factor $\sigma^B$ [16]. This group includes the sRNA Rli47, a ~500 nucleotide-long sRNA located in the intergenic region between *lmo2141* and *lmo2142*. Transcriptomic studies identified a $\sigma^B$-dependent promoter upstream the *ril47* transcript, also referred to as *sbrE* (sigma-B-dependent RNA) in the literature, and a Rho-dependent terminator at its 3′-end [17–19]. It is expressed at higher levels in *L. monocytogenes* stationary phase cells [18–20], in the intestinal lumen [18], in macrophages [17] and in soil environments in mutants lacking the AgrA regulator [21]. Although studies on Rli47 function have identified altered transcription of a number of genes including lmo0636, lmo0637 (methyltransferase) and lmo2094 (L-fuculose-phosphate aldolase) in a mutant lacking this sRNA [20], no growth or survival phenotype under a variety of different environmental conditions has been identified and its function has therefore remained undefined.

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Here we report predicted binding targets for Rli47 using an in silico and in vivo analysis of its secondary structure, and we confirmed its binding in vitro to a target mRNA associated with branched-chain amino acid (BCAA) biosynthesis. The data presented show that Rli47 can base-pair through a single-stranded CU-rich loop with the Shine-Dalgarno (SD) region of the ilvA mRNA, which encodes threonine deaminase (TD), the first key enzyme in the L-isoleucine (ile) biosynthetic pathway that catalyzes the deamination of L-threonine to produce alpha-ketobutyrate \[ \text{22,23} \]. This interaction negatively regulates the expression of ilvA, reducing the threonine deaminase enzymatic activity. Global transcriptional profiling confirmed the role of Rli47 in modulating amino acid metabolism and in particular influencing the CodY regulon. Together, our data support a model where $\sigma^B$, CodY and Rli47 form a regulatory network that serves to repress isoleucine biosynthesis even in conditions where isoleucine is depleted from the environment.

Results
Defining the sequence and structure of rli47 sRNA

The rli47 gene is located on the intergenic region between lmo2141 and lmo2142 of L. monocytogenes EGD-e (Figure 1(a)). Nucleotide BLAST searches [24] against other Listeria species for which whole genome sequence is available revealed that rli47 is present in all Listeria sensu stricto species (L. monocytogenes, L. innocua, L. seeligeri, L. ivanovii, L. welshimeri and L. marthii) but was not found in the genomes of species of the Listeria sensu lato group (L. weihenstephanensis, L. fleischmannii, L. floridensis, L. aquatica, L. newyorkensis, L. cornelensis, L. grandensis, L. ripartii, L. booriei, L. rocourtiae).

The rli47 gene belongs to the $\sigma^B$ regulon and a $\sigma^B$-dependent promoter has been identified upstream from the predicted first nucleotide (Fig. S1A) [17,19]. Earlier studies claimed different sizes for this sRNA: 314 nt [17], 446 nt [25], 509 nt [18] and 514 nt [19]. Additionally, a Rho-independent terminator was previously identified at the 3'-end of the transcript. This terminator is shared with the flanking gene lmo2142 located in the opposite strand [19]. Besides the differences in length, the reported transcription start and stop sites also differed between research groups. Therefore, in order to proceed with the construction of a deletion mutant lacking the entire transcript we mapped rli47 on the genome of L. monocytogenes EGD-e to help better assign its start and stop sites.

Using northern blot analysis (Fig. S1B), primer extension (Fig. S1C) and secondary structure predictions (Figures 1(b and c)), Rli47 was estimated to be 515 nt in length (from nucleotides 2,226,036A to 2,226,550A in the genome of L. monocytogenes EGD-e). Computational (Figure 1(b)) and in vivo (Figure 1(c)) predictions of the secondary structure of Rli47 showed that it is likely to fold as a six stem-loop structure. These predicted six stem-loop structures were labelled from A to F; (A) 42G – 74U, (B) 112C – 185U, (C) 201C – 268G, (D) 279G – 382C, (E) 401U – 424A and (F) 474G – 513U (Figure 1(b)). The six stem-loop structures

![Figure 1. Mapping and folding structure of Listeria monocytogenes Rli47. (a) Mapping rli47 on the genome of Listeria monocytogenes EGD-e. A $\sigma^B$-dependent promoter (Pb) is represented, and the lollipop structure denotes a hypothetical transcription terminator structure shared by rli47 and lmo2142. (b) Folding structure was predicted in silico by mfold. The six stem-loop structures were labelled from A to F; (A) 42G – 74U, (B) 112C – 185U, (C) 201C – 268G, (D) 279G – 382C, (E) 401U – 424A and (F) 474G – 513U (Figure 1(b)). The six stem-loop structures were labelled based on their position relatively to the in silico structure, as derivative structures were named A' and E'.](image-url)
produced by the DMS-MaPseq in vivo mapping approach (Materials and Methods) showed a similar assembly, although the first and fifth stem-loops were structured in different stretches; (A’) 39U – 81G, (E’) 441U – 470A (Figure 1(c)). Thus, the secondary structures predicted by both the in vivo and in silico approaches are in good agreement.

Regulatory sRNAs of Gram-positive bacteria often contain single-stranded CU-rich motifs, which are important for target mRNA interactions by base-pairing with the SD sequences \[26–31\]. Analysing the in silico and in vivo generated Rli47 stem-loop structures in detail identified a CU-rich motif on the single-strand of the stem-loop C, making this a promising region for base-pairing with target mRNAs. Furthermore, Rli47 is unlikely to interact with the RNA-binding protein Hfq since the level of Rli47 was not affected by its absence \[18\].

**σB-dependent transcription of rli47 is induced during exponential growth in DM**

To investigate the conditions that lead to Rli47 transcription, the levels of the sRNA were determined via northern blot analysis on total RNA purified from L. monocytogenes EGD-e in both complex medium TSB (Tryptic Soy Broth) and a minimal medium DM (chemically Defined Medium). To further investigate \(σB\)-dependency of the rli47 transcription, a \(σB\) deletion mutant strain (Δ\(σB\)) was included in this analysis. An rli47 deletion mutant strain (Δrli47) was constructed that lacked the whole rli47 coding sequence except the hypothetical terminator structure shared with the downstream gene lmo2142 (Fig. S1). This deletion mutant was used as a negative control for the transcript measurements. RNA samples used for northern blots were extracted from cells harvested at exponential and stationary phase cells growing in DM and TSB, which were loaded in a gel per growth stage and probed for Rli47 and rRNA 16S (Fig. S2). Rli47 RNA was detected in the wild-type (WT) in both exponential and stationary phase in both growth media, DM (Figure 2) and TSB (Fig. S3). As expected, Rli47 was not detected in the Δrli47 mutant. Transcription was highly \(σB\)-dependent in both media although some \(σB\)-independent transcription was detected in TSB at exponential phase (Fig. S3). The deletion of rli47 had minimal impact on the transcription of the upstream (lmo2141) and downstream (lmo2142) genes, as determined by RT-PCR on cells grown in either DM to exponential phase or TSB to stationary phase (Fig. S4).

**Rli47 binds to the SD region of the ilva mRNA**

We hypothesized that this Rli47 might act in trans, via direct binding to the target mRNAs, leading to inhibition of translation initiation and/or influencing transcript stability. To explore this possibility, we first performed in silico analyses of the potential base-pairing between the sRNAs and mRNAs. Several mRNAs were computationally predicted to bind to Rli47 using IntaRNA \[32–34\]. In order to narrow the list to the most plausible targets, the following criteria were applied.
to the predicted binding regions: (1) the interaction should involve a single-stranded region of the sRNA; (2) it should include the SD sequence of the target mRNA; (3) the interaction should have a binding energy greater than 16 kJ mol\(^{-1}\).

Based on these constraints, \(copp\), \(ribCF\), \(ilvA\) and \(addB\) mRNAs were identified as putative targets of Rli47 (Table 1).

To further investigate the binding of Rli47 to these mRNAs, Electrophoretic Mobility Shift Assays (EMSAs) were performed (Figure 3), where full-length labelled sRNA was incubated with increasing concentrations of 5’-end unlabelled RNA of \(ilvA\) (99 nt), \(copB\) (75 nt), \(ribCF\) (77 nt) or \(addB\) (183 nt). For this, RNA fragments corresponding to the region of interest were synthesized, including the entire intergenic region plus at least 20 to 30 nt of the coding region. The electrophoretic mobility of Rli47 was clearly shifted when unlabelled \(ilvA\) RNA was used in the assay, suggesting their interaction (Figure 3). However, no binding was detected between Rli47 and \(copB\), \(ribCF\) or \(addB\) under the same experimental conditions (Figure 3). These results demonstrate a likely interaction between Rli47 and the \(ilvA\) mRNA.

Interestingly, the SD sequence of \(ilvA\) mRNA was predicted to bind to the CU-rich single-stranded region of the third stem-loop (stem-loop C) of the sRNA (Fig. 1B and 4A). To investigate which part of Rli47 was responsible for this interaction, a truncated version of Rli47, from the transcription start site to nucleotide 276C, which lies after the predicted third stem-loop as a likely location of the interaction. In order to investigate the importance of the CU-rich region of the third stem-loop in the interaction with \(ilvA\) mRNA, a mutated version of Rli47 (sRli47\(^{GAGG\rightarrow CACC}\)) was synthesized, where nucleotides 233 to 236 were altered from \(CCUC\) to \(GGUG\) in the truncated version of Rli47. This alteration was predicted to prevent the interaction of the Rli47 CU-rich loop with the SD region of the \(ilvA\) transcript (Figure 4A). The Rli47 derivative was labeled, mixed with increasing concentrations of \(ilvA\) RNA and binding was analyzed in an EMSA (Figure 4C). Since Rli47 migrated as two bands in gel electrophoresis, even in the unbound state (Figure 4), it suggests that the sRNA can adopt two alternate structures under the conditions used in the binding experiments. Nonetheless, the possibility that the presence of the two bands could also result from an abiotic transcription cannot be ruled out. To further confirm this interaction, a complementary mutation was introduced into the \(ilvA\) transcript changing the predicted SD region from GAGG to CACC, and the construct was designated \(ilvA\text{-SD}^{GAGG}\text{CACC}\) (Figure 4A). Differences in the labelling efficiencies between sRli47 and sRli47\(^{GAGG\rightarrow CACC}\) were consistently observed (Figure 4 and S5). In silico analysis predicted several RNA structures with similar folding patterns for both sRli47 and sRli47\(^{GAGG\rightarrow CACC}\) (AG ranging from \(-66.10\) to \(-63.30\) kcal/mol). The single-stranded loop, where the mutated region resides, was preserved in all of the proposed mfold structures, implying that the differences in labelling efficiencies are most likely due to changes in the tertiary structure of the sRNA species. Further secondary structure predictions on \(ilvA\) and \(ilvA\text{-SD}^{CACC}\) mRNAs revealed that the mutation caused differences in folding. Thereafter, to minimize the putative effect of such differences in secondary structures, both RNAs were heated and cooled together for the mutational analysis, ruling out any concerns regarding structural differences on the mutant RNA secondary structures relative to the WT versions (Figure 4C). The results revealed that mutations in the CU-rich sequence of the loop prevented the interaction with \(ilvA\) RNA.

**Rli47 CU-rich loop is responsible for binding to SD regulatory region of ilvA mRNA**

To further define the base-pairing between the sRNA Rli47 and \(ilvA\) mRNA we focused on the CU-rich region of the predicted third stem-loop as a likely location of the interaction. In order to investigate the importance of the CU-rich region of the third stem-loop in the interaction with \(ilvA\) mRNA, a mutated version of Rli47 (sRli47\(^{GAGG\rightarrow CACC}\)) was synthesized, where nucleotides 233 to 236 were altered from \(CCUC\) to \(GGUG\) in the truncated version of Rli47. This alteration was predicted to prevent the interaction of the Rli47 CU-rich loop with the SD region of the \(ilvA\) transcript (Figure 4A). The Rli47 derivative was labeled, mixed with increasing concentrations of \(ilvA\) RNA and binding was analyzed in an EMSA (Figure 4C). Since Rli47 migrated as two bands in gel electrophoresis, even in the unbound state (Figure 4), it suggests that the sRNA can adopt two alternate structures under the conditions used in the binding experiments. Nonetheless, the possibility that the presence of the two bands could also result from an abiotic transcription cannot be ruled out. To further confirm this interaction, a complementary mutation was introduced into the \(ilvA\) transcript changing the predicted SD region from GAGG to CACC, and the construct was designated \(ilvA\text{-SD}^{GAGG\rightarrow CACC}\) (Figure 4A). Differences in the labelling efficiencies between sRli47 and sRli47\(^{GAGG\rightarrow CACC}\) were consistently observed (Figure 4 and S5). In silico analysis predicted several RNA structures with similar folding patterns for both sRli47 and sRli47\(^{GAGG\rightarrow CACC}\) (AG ranging from \(-66.10\) to \(-63.30\) kcal/mol). The single-stranded loop, where the mutated region resides, was preserved in all of the proposed mfold structures, implying that the differences in labelling efficiencies are most likely due to changes in the tertiary structure of the sRNA species. Further secondary structure predictions on \(ilvA\) and \(ilvA\text{-SD}^{CACC}\) mRNAs revealed that the mutation caused differences in folding. Thereafter, to minimize the putative effect of such differences in secondary structures, both RNAs were heated and cooled together for the mutational analysis, ruling out any concerns regarding structural differences on the mutant RNA secondary structures relative to the WT versions (Figure 4C). The results revealed that mutations in the CU-rich sequence of the loop prevented the interaction with \(ilvA\) RNA,

### Table 1. Interactions between target mRNAs and Rli47 predicted in silico. Predictions were obtained by the IntaRNA software [32–34]. Top four hypothetical targets which binding prediction fulfilled the selection criteria: (i) Energy > 16 kJ mol\(^{-1}\); (ii) Single-strand region on Rli47; (iii) Overlap the Shine-Dalgarono region on the target mRNA. No \(\sigma\) promoter was found in silico in any of the target genes [25]. TSS = transcription start site.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Energy (kJ mol(^{-1}))</th>
<th>mRNA binding region (nt from start codon)</th>
<th>Rli47 binding region (nt from TSS)</th>
<th>Molecular function</th>
<th>Biological process</th>
</tr>
</thead>
<tbody>
<tr>
<td>copB</td>
<td>19.74</td>
<td>-13 to 18</td>
<td>259 to 292</td>
<td>Adenosylcobinamid-phosphate synthase</td>
<td>Cobalamine biosynthetic process</td>
</tr>
<tr>
<td>ribCF</td>
<td>17.57</td>
<td>-11 to 14</td>
<td>262 to 286</td>
<td>Guanyltransferase</td>
<td>Riboflavin biosynthetic process</td>
</tr>
<tr>
<td>ilvA</td>
<td>16.49</td>
<td>-9 to 17</td>
<td>219 to 241</td>
<td>Bifunctional flavokinase, FAD synthetase</td>
<td>Isoleucine biosynthetic process</td>
</tr>
</tbody>
</table>

**Figure 3.** Rli47 and hypothetical target mRNAs base-pairing by Electrophoretic Mobility Shift Assay. Labelled Rli47 full-size transcripts were incubated with increasing concentrations of unlabelled \(ilvA\), \(addB\), \(ribCF\) or \(copB\) mRNA. The fraction of unbound Rli47 is shown below each lane.
suggesting that this region is crucial for the binding of Rli47 to ilvA transcripts (Figure 4(c)). Similar results were observed in non-denatured RNAs (Fig. S5). The mutated version of ilvA (ilvA-SDCAC) did not interact with the wild-type version of Rli47 (Figure 4(c)). When the complementary mutated versions of ilvA RNA (ilvA-SDCAC) and Rli47 mutated CU-rich loop sequence (sRli47GGUG) were mixed, the interaction was restored (Figure 4(c)). These results confirmed that the CU-rich sequence of the third stem-loop of Rli47 is required for base-pairing to the SD region of ilvA mRNA and thus identified the region that is critical for the interaction.

Deletion of sigB and Rli47 increases ilvA mRNA

The in vitro interaction of Rli47 with the SD region of the ilvA mRNA suggested a regulatory role of the sRNA and we sought to investigate this in vivo. To this end, we performed RT-qPCR on total RNA purified from L. monocytogenes EGD-e wild-type, ΔsigB and ΔRli47 exponential phase cells grown in DM and stationary phase cells grown in TSB, conditions where the levels of Rli47 were previously shown to be highest (Figure 2 and S3). The levels of Rli47 and ilvA mRNA were quantified in the knockout cells relative to WT cells and normalized to 16S rRNA transcript levels. In both growth conditions, Rli47 levels were lower in the ΔsigB mutant (log₂ FC = −7.45 in DM and log₂ FC = −10.94 in TSB) and essentially undetectable in the Δrli47 strain (log₂ FC = −16.67 in DM and log₂ FC = −18.21 in TSB) relative to the wild-type. In contrast, the ilvA transcript was present at an elevated level in both ΔsigB mutant (log₂ FC = 2.46 in DM and log₂ FC = 5.37 in TSB) and Δrli47 mutant (log₂ FC = 1.05 in DM and log₂ FC = 7.44 in TSB) strains (Figure 5 and S6). However, in DM, the difference in the ilvA transcript levels in the Δrli47 strain relative to the WT was not statistically significant (p-value = 0.67). Although
Rli47 transcript levels were lower in both knockout mutants, a significant difference in Rli47 mRNA level was shown, suggesting that some transcripts were still being produced in the ΔΔilvA strain. This provided further evidence that Rli47 transcription is largely but not solely dependent on σB. The difference in the ilvA relative transcription between mutant strains was not significant (p-value > 0.05) for cells grown in DM (Figure 5), however in TSB there was significant difference (Fig. S6), suggesting that σB has rli47-independent effects on ilvA mRNA levels. Together these data indicate that Rli47 has a negative effect on the level of ilvA transcripts in vivo, at least in the complex medium TSB, suggesting that the interaction detected in vitro is physiologically significant in vivo.

Both rli47 and σB cause L-isoleucine auxotrophy by decreasing TD activity

The ilvA gene encodes threonine deaminase (TD), which is the first key enzyme on the L-isoleucine biosynthetic pathway that catalyzes the deamination of threonine to produce α-ketobutyrate, water and ammonia. To investigate the contribution of Rli47 to L-isoleucine biosynthesis, L. monocytogenes EGD-e wild-type, ΔσB and ΔΔilvA strains were grown in DM either with (DM) or without (DM-ile) isoleucine supplementation (Figure 6(a)). No differences in growth lag or lag time were observed among strains grown in DM (Table S3). However, the ΔΔilvA strain had a significantly shorter lag phase than the WT during growth in DM-ile (Figure 6(a)), as well as in media with reduced levels of isoleucine (Table S3 and Fig. S7). This phenotype was reversed when the ΔΔilvA deletion was complemented in trans with a native copy of the rli47 (rli47-c) gene including its own promoter region (Figure 6(a)). The rli47-complemented strain shows a longer lag time than the WT in DM-ile medium. In these growth experiments, the ΔΔilvA mutant displayed a phenotype similar to the ΔΔilvA mutant but had an even shorter lag time (Figure 6(a)). These data suggest that σB and Rli47 activities delay the onset of growth under isoleucine limiting conditions, consistent with the hypothesis that Rli47 negatively regulates expression of ilvA.

To determine if the elevated ilvA mRNA levels found in the ΔΔilvA and ΔΔilvA mutant backgrounds resulted in a corresponding effect on the levels of TD, its activity was measured in total protein extracts from L. monocytogenes EGD-e wild-type, ΔΔilvA and ΔΔilvA grown to stationary phase in both DM and DM-ile (Figure 6(b)). While low levels of TD were detected in the WT grown in DM, significantly higher activity was recorded in the ΔΔilvA mutant, but the increase in the ΔΔilvA was not significant. Isoleucine depletion did not affect TD activity in the WT, but it induced a significant increase of TD activity in both ΔΔilvA and ΔΔilvA mutants (p = 0.024 and p < 0.0001, respectively, for ΔΔilvA and ΔΔilvA mutant strains) (Figure 6(b)). Consistent with the growth lag data in DM-ile (Figure 6(a)), the activity of TD was significantly higher in the ΔΔilvA mutant than in the ΔΔilvA mutant strain. Altogether, these results strongly suggest that Rli47 plays a role in the impairment of isoleucine biosynthesis through repression of ilvA expression, even in media where this amino acid is absent. They further suggest that additional σB-dependent mechanisms leading to TD repression are also present (Figure 6(c)).

Transcriptomic analysis shows that amino acid transport and metabolism is affected by rli47 deletion

To complement the in silico and in vitro data described above, and investigate the overall impact of Rli47 on gene transcription, a transcriptomic analysis of L. monocytogenes ΔΔilvA mutant and the parental strain EGD-e was performed using RNA-seq during growth at 37°C in DM, a condition where Rli47 is strongly expressed in the WT (Figure 2). Significant changes in the transcript levels of 155 genes were detected between the two strains (Figure 7(a)). The relative abundance of each COG category in the set of differentially transcribed genes was determined. Overall, genes involved in amino acids transport and metabolism were highly represented in both upregulated and downregulated sets of genes, suggesting a direct and/or indirect role of Rli47 in modulating amino acid metabolism (Figure 7(b)). A set of 105 genes showed higher transcript levels in the ΔΔilvA mutant. The gene with the highest fold change was lmo1634 (Fold-change = 29.9). It encodes a bifunctional acetaldehyde-CoA/alcohol dehydrogenase involved in pyruvate metabolism. Three pyruvate-formate lyase encoding genes were found upregulated as well (pfaA, pfcC and pfbB). Transcripts of genes encoding transcription regulators (lmo2365, lmo2364 and lmo2447), in ferrous iron transport (fomA and fomB), (R)-2-hydroxyglutaryl-CoA dehydratase (yijJ), phenazines (lmo2637), mosol degradation into acetyl-CoA (iol), anaerobic bacterial respiration (lmo0355), arginine biosynthesis (argG) and valine/isoleucine biosynthesis (ilvD) were all found in the top 20 most highly expressed genes (Table S4 and Figure 7(a)). Thirteen sRNAs were in the set of genes with higher transcript level, of which Rli60 (Fold-change = 2.8) was the only figuring in the top 20 most
upregulated genes in the Δrli47 mutant strain. Interestingly, rli60 belongs to the ilv-leu BCAA biosynthesis operon (rli60, ilvD, ilvB, ilvN, ilvC, leuA, leuB, leuC, leuD and ilvA). This sRNA has recently been identified as a riboregulator regulating the transcription of the ilv-leu operon depending on isoleucine levels [12]. Four of the genes (rli60, ilvD, ilvB and ilvN) of this operon were in the set of genes with higher transcript levels (Fold-changes of 2.8, 2.7, 2.00, 1.8, respectively), but the increased levels detected for the last six genes of the operon (ilvC, leuA, leuB, leuC, leuD and ilvA) did not reach the significance threshold (Fold-change = 1.4, 1.3, 1.2, 1.1, 1.1 and 1.2, respectively). Fifty genes including four sRNAs showed lower transcript levels (Table S4). The most downregulated gene in the Δrli47 mutant was copB (Fold-change = −3.4), one of the in silico predicted Rli47 targets (Table S4) and which encodes adenosylcobinamide-phosphate guanylyltransferase, a bifunctional cobalamin biosynthesis protein. Another gene that was significantly downregulated was cbiD (cobalt percocin-6A synthase), which is also involved in cobalamin biosynthesis. In the top 20 most downregulated genes were genes encoding proteins involved in late competence (lmo1341), secretion (esuC), propanediol dehydratase reactivation (lmo1157), ABC transporter systems (lmo0861 and lmo0135), glycine cleavage (gcvT and gcvPA), phosphate transport system (lmo2497), uroporphyrinogen-III methyltransferase/synthase (lmo1201) and de novo purine biosynthesis (lmo1201) and de novo purine biosynthesis (purH, purN and purM) (Figure 7(a) and Table S4). However, the function of some other genes (lmo2807, lmo2161, lmo0702 and lmo0461) remains unknown as all encode hypothetical proteins. Four sRNAs were in the set of genes with lower transcript levels in the Δrli47 mutant strain. The role of Rli89 is undetermined while SbrA is a highly σB-dependent sRNA that is likely to be involved in the fine-tuning expression of genes involved in stress response, metabolism and virulence [18,19,35]. Some overlap with AgrA, σB, CodY and PrfA regulons was recorded (Table S5). The overlap with CodY regulon was the largest (n = 42 genes), and most of the overlapping genes belong to the amino acids transport and metabolism functional category. Indeed, in L. monocytogenes CodY controls the transcription of genes involved in metabolism, stress responses, motility and virulence in response to the availability of BCAA [10]. Since isoleucine serves as ligand for modulating CodY activity, deregulation of ilvA expression in the
mutant likely accounts for the high proportion of ilv-leu and acts as a ribosome-
Expression analysis of genes differentially transcribed by (mutant, suggesting that the levels of the entire operon, which deletion mutant (was deleted from the EGD-e (NC_003210.1). Right upper pie chart, operon, 0.58> L. monocytogenes rli47 C. M. MARINHO ET AL.

There may also be ilvDBN operon in behaves as a partial auxotroph for BCAA, strategy, whereby the pathogen could couple virulence operon transcript are lower in the presence of Rli47. It seems unlikely that this effect occurs at the transcriptional level since transcription is controlled by the combined actions of CodY and the transcriptional attenuator Rli60 which is located upstream from ilvD and acts as a ribosome-dependent transcriptional attenuator of the operon [12]. More likely the binding of Rli47 to the SD region of ilvA, negatively affects the overall stability of the mRNA. Thus, the recent model presented by Brenner and co-workers [12] describing the regulation of the ilv-leu operon in L. monocytogenes has acquired an additional layer, with Rli47 providing another level of negative control that operates to restrict isoleucine biosynthesis even when this amino acid is absent from the growth medium.

The question of why L. monocytogenes would repress the biosynthesis of an amino acid it requires for growth is not easily answered. It has been known for many years that L. monocytogenes behaves as a partial auxotroph for BCAA, requiring supplementation of these amino acids into the growth medium in order to achieve optimal growth [12,36,37], despite having the genes required for their biosynthesis [38]. Here we provide evidence suggesting that Rli47 contributes to the repression of the ilv-leu operon, which encodes the genes necessary for BCAA biosynthesis, and show that growth is improved in a defined medium lacking isoleucine when this sRNA is absent (Figure 6). Interestingly, a similar phenotype was reported by Brenner et al. (2018) for L. monocytogenes 10403S when the rli60 was deleted from the genome. It encodes a leader peptide that is rich in ile/val/leu codons which serves to control transcriptional termination in a manner that is dependent on the availability of BCAA in the medium, a response that is mediated by the translation rate of the leader peptide [12]. The authors of this study proposed that this regulation of BCAA might represent a host adaptation strategy, whereby the pathogen could couple virulence gene expression with the levels of BCAA encountered in micro-niches within the host. In this model, the link between BCAA and virulence gene expression is made by CodY which positively regulates PrfA expression in response to low BCAA levels [9,12,39] and induces CodY which positively regulates PrfA expression in response to low BCAA levels [9,12,39]. While this model is certainly worth exploring further it is not yet clear how an additional layer of negative regulation, mediated by the sRNA Rli47, can be incorporated into this view. One possibility is that the stresses encountered within the host, particularly in the gastrointestinal tract where sRNA Rli47 is known to be active [18], trigger this additional layer of negative control. This Rli47-mediated effect could serve as an early stimulus to prime cells for
virulence by inducing prfA transcription in response to σB activation, but further studies will be needed to explore this idea fully.

An alternative model that seems worth considering is the possibility that restricted BCAA biosynthesis may serve to limit the growth of *L. monocytogenes* when it is under stress, thereby enhancing its survival. This possibility is suggested by a number of observations, the first of which is that *rli47* transcription is under the control of σB, the regulator of the general stress response [7,40–42], and so it would be unsurprising if it played a role in stress survival. Secondly, a number of studies have highlighted the fact that mutants lacking sigB grow at a faster rate than the WT under some culture conditions [43–46], suggesting that σB acts to limit growth under some conditions. A recent study on the intracellular behaviour of *L. monocytogenes* reports that a subpopulation can exist in a non-growing state within vacuoles in mammalian cells [47], although the mechanism for this phenomenon has yet to be identified. In several other bacterial species, the presence of dormant persister cells within the population has been shown to contribute to increased antibiotic and stress resistance, and a variety of different mechanisms contribute to this persister state. Indeed, this phenomenon appears to be ubiquitous amongst bacteria although in many cases the underlying mechanisms are unknown [48–50]. Thus, we speculate that tight negative control of BCAA biosynthesis under conditions where cells are subject to stress might serve to block growth and confer an increased resistance that is associated with non-growing cells, even under conditions when the supply of BCAA in the medium is limiting. This effect could contribute to survival in both terrestrial and host-associated niches and is consistent with the finding that this sRNA is present in non-pathogenic species of *Listeria*.

Interestingly the effect of the Δ*rli47* deletion on growth in DM with limiting BCAA was apparently confined to the lag phase (Figure 6 and S7). Once cells began to grow the growth rates were not significantly different in this medium (Table S3), whether this sRNA was present or not. Since Rli47 is expressed strongly under conditions where σB is highly active (Figures 2 and S3) [18,19], it is possible that Rli47 serves to limit the exit from lag phase under stressful conditions in order to allow a period of protection and repair prior to renewed growth. The question of how the levels of Rli47 are modulated during this transition from lag to active growth will require further investigation, but it could simply occur when σB activity drops due to the presence of more benign prevailing conditions and subsequent loss of Rli47 through normal RNA degradation processes.

The size of Rli47 and the complexity of its secondary structure points to other possible roles for this sRNA other than its involvement in regulating BCAA biosynthesis. The transcriptomic analysis presented here was undertaken to complement the more targeted approaches that we used to identify binding targets for Rli47. Strikingly one of the potential binding targets predicted in silico, *copB*, was found to be significantly affected in the Δ*rli47* mutant, where it was the transcript showing the largest reduction compared to the WT (Figure 7 and Table S4). While the appearance *copB* in the RNA-seq data set does not provide evidence of a direct interaction between it and Rli47 this certainly remains a distinct possibility. It could be that the in vitro binding conditions...
used for the EMSA analysis fail to emulate the conditions within the cytoplasm in some crucial aspect, for example, if additional factors such as RNA chaperones are required to permit successful stable interactions, or if the interactions occur very transiently.

In any case, it is interesting to observe that cspB and cblD, both involved in cobalamin (vitamin B₁₂) biosynthesis, as well as genes involved in the B₁₂-dependent pathway for 1,2-propanediol utilisation (ioll and lmo1157) were all significantly affected in the absence of Rli47. These genes are only present in Listeria sensu stricto species that can survive within the gastrointestinal tract, the so-called Listeria sensu stricto species that include both the pathogenic species L. monocytogenes, L. ivanovi [51], and commensal species L. innocua, L. marthii, L. welshimeri and L. seeligeri [52]. Interestingly Rli47 is also confined to the genomes of these species and is not found in the Listeria sensu lato species, a clade that includes species only isolated from non-host-associated environments (data not shown) [52,53].

These observations lead us to speculate that Rli47 may contribute to modulating the expression of genes that are required for survival within the environment of the gastrointestinal tract.

Overall this study identifies a defined role for an sRNA that is known to be under σ⁸ control and induced within the mammalian gastrointestinal tract but whose function has remained elusive. It is now one of the small number of sRNAs in L. monocytogenes where a clear function has been established. The finding that it acts to repress BCAA biosynthesis implicates it as a global regulator of transcription in this pathogen because of its influence on isoleucine levels, the ligand that determines the activity of the global regulator CoDY. The evolutionary rationale for supressing the biosynthesis of isoleucine remains to be fully determined, but we favour a model that involves growth restriction under harsh conditions, contributing to the survival of this adaptable pathogen in niches both outside and within its mammalian host.

Materials and methods

Bacterial strains, plasmids and primers

In this study, Listeria monocytogenes EGD-e was used as the wild-type strain [38]. The isogenic mutant derivatives of this strain were constructed using standard techniques for DNA manipulation in L. monocytogenes [54]. An rli47 deletion mutant was obtained by allelic replacement of the wild-type gene by homologous recombination using the pMAD shuttle suicide plasmid as previously described [55]. The gene deletion construct was synthesised by Invitrogen GeneArt Gene Synthesis (Thermo Fisher Scientific). The deletion insert (552 bp) spanning 258 bases upstream of the TSS of rli47 to 209 bases downstream of the hypothetical terminator structure (Fig. S1), containing a deletion of 439 bp (from base 18 to 456) in the rli47 gene, was sub-cloned in the pMAD vector and confirmed by DNA sequencing. Alternatively, a sigB knockout mutant, including a 561 bases deletion (from base 64 to 624) was constructed using the pMAD shuttle plasmid [55], and confirmed by DNA sequencing. Whole-genome sequencing was performed by Illumina sequencing (MicrobesNG) and genomes analyzed by Bresq [56]. Sequencing indicated additional point mutations in lmo2761 (G1063→T) in the sigB deletion mutant and two in the rli47 deletion mutant, lmo1255 (G1156→A) and lmo2142 (T327→A). A complement of rli47 deletion was constructed by cloning a PCR fragment, spanning approximately 91 bases upstream of the TSS of rli47 to a region lying just downstream of the hypothetical terminator, into the pMK4 vector [57], and transform into Listeria monocytogenes Δrli47 competent cells by electroporation. Primers used for in-frame deletions are listed in Table S1. All plasmids and strains used in this study are listed in Table S2.

Growth conditions

L. monocytogenes was routinely grown at 37°C with aeration in tryptic soy broth (TSB, Conda) as a rich medium or in a chemically defined medium (DM) for L. monocytogenes. Alternatively, strains were grown in brain-heart infusion broth (BHI, Lab M) or Luria-Bertani broth (LB; Sigma Aldrich) supplemented with 50 mM glucose, when stated. DM was prepared as described previously [58]. When appropriate, cultures were supplemented with chloramphenicol (10 μg ml⁻¹), ampicillin (100 μg ml⁻¹), kanamycin (50 μg ml⁻¹), erythromycin (5 μg ml⁻¹) or L-threonine (0.1 μg ml⁻¹). For growth under limiting concentrations of isoleucine (ile), DM was freshly made with 10-fold less of isoleucine (resulting in a final concentration of 10 μg ml⁻¹ or 80 μM), 100-fold less of isoleucine (resulting in a final concentration of 1 μg ml⁻¹ or 8 μM), or completely depleted of isoleucine. For growth curves, bacteria from overnight DM cultures were washed 3 times with PBS and adjusted to OD₅₉₀ of 0.05 in fresh DM without ile or supplemented with 100, 10 or 1 μg ml⁻¹ of ile (800, 80 or 8 μM ile, respectively). OD₅₉₀ measurements were taken every 2 h. Lag and Doubling times were calculated by GrowthRates 3.0 [59].

Primer extension

Primer extension experiments were performed as previously described [60]. A ΔsigB strain was used as a negative control, given the σ⁸-dependency of rli47 transcription [17], in opposite to a ΔagrA strain where Rli47 was expected to be highly expressed [21]. RNA was extracted from stationary phase cells grown at 37°C in both complex media BHI and LB + Glucose.

In silico predictions

The intaRNA software [32–34] was used for predicting interactions between target mRNAs and Rli47. RNA–RNA interaction search was performed using the full-length Rli47 sRNA sequence as a query against the RNA sequences from L. monocytogenes EGD-e genome (NC_003210). The following levels of stringency were applied: (1) sequences spanning 75 nt upstream to 75 nt downstream the start codon of target genes, (2) one (sub)optimal interaction output overlap in query, (3) minimum of 7 basepairs in seed, (4) output including only interactions with a delta energy ≤100 kcal mol⁻¹, (5)
Modelling of rli47 secondary structure in vivo

The data on dimethyl sulphate (DMS) reactivity of Rli47 RNA were imported from NCBI’s Gene Expression Omnibus (GEO accession number GSE118387). In that study, DMS reactivity of L. monocytogenes non-coding RNAs was probed using the DMS-MAseq protocol during mid-exponential growth at 37°C in BHI medium [62]. DMS methylates unpaired adenines (A) or cytosines (C) but not A or C engaged in pairing. In brief, DMS (3%) was added to growing cells for 3 min before quenching and RNA-preparation. cDNA of the RNA were synthesized using TGIRTIII reverse transcriptase which incorporates Baseline-Zero DNase (Epicentre) in the presence of RiboLock Ribonuclease inhibitor (40 U µl⁻¹) (Thermo Fisher Scientific) for 30 min at 37°C followed by purification using Zymo-Spin column (ZymoResearch). Briefly, sample was mixed with 2 volumes of RNA-Binding Buffer and added to an equal volume of ≥99.8% ethanol. The mixture was vortexed and transferred to Zymo-Spin™ IC Column and centrifuged at 12,000 x g. The RNA bound to column was washed twice with RNA Wash Buffer then RNA was eluted in DNase/Rnase free water. Concentration of RNA was measured using fluorescence-based Qubit™ RNA HS Assay (Thermo Fisher Scientific).

Ribosomal RNA depletion and library preparation

To enrich mRNA and remove ribosomal RNA (rRNA) from total RNA, total RNA was treated with Ribo-Zero rRNA removal kit (Illumina). Briefly, beads were washed twice and hybridized with probes at 68°C for 10 min. Total 500 ng RNA was added to the mixture and incubated at RT and 50°C for 5 min each followed by separation of mRNA from rRNA which was bound to the beads using magnetic stand. Enriched mRNA was purified by Zymo-Spin column (ZymoResearch) and run on Labchip GX II bioanalyzer (Perkin Elmer) to confirm depletion of rRNA. Preparation of cDNA fragment libraries was performed using the NEBNext™ Ultra™ II Directional RNA Library Prep Kit for Illumina® (Illumina) without slight modifications. Briefly, the enriched mRNA was fragmented for 15 min at 94°C and reverse transcribed to synthesize the first-strand cDNA followed by second strand cDNA synthesis. Double-stranded cDNA (ds cDNA) was purified using NucleoMag (Macherey nagel) SPRI selection. End repair was performed on the ds cDNA library followed by ligation of adaptors. After purification using NucleoMag SPRI beads, test RT-qPCR (Applied Biosystems) was performed using KAPA HiFi polymerase (Roche) with Evagreen® (Biotium) to determine appropriate cycle numbers for PCR. Using NEBNext Multiplex Oligos for Illumina (Dual Index Primers), high fidelity PCR was performed using KAPA HiFi polymerase to selectively enrich library fragments. The PCR

RNA integrity, quantification and DNase I treatment

Three independent biological replicates of RNA samples extracted from L. monocytogenes EGDe and Δrli47 were stored at −80°C before treatment. The quality of RNA was assessed using Labchip GX II bioanalyzer (Perkin Elmer). To remove DNA contamination, total RNA was incubated with Baseline-Zero DNase (Epipcent) in the presence of RiboLock RNase inhibitor (40 U µl⁻¹) (Thermo Fisher Scientific) for 30 min at 37°C followed by purification using Zymo-Spin column (ZymoResearch). Briefly, sample was mixed with 2 volumes of RNA-Binding Buffer and added to an equal volume of ≥99.8% ethanol. The mixture was vortexed and transferred to Zymo-Spin™ IC Column and centrifuged at 12,000 x g. The RNA bound to column was washed twice with RNA Wash Buffer then RNA was eluted in DNase/RNase free water. Concentration of RNA was measured using fluorescence-based Qubit™ RNA HS Assay (Thermo Fisher Scientific).
products were purified twice using NucleoMag (Macherey
nagel) SPRI beads and the quality of the final library was
assessed on Labchip GX II bioanalyzer (Perkin Elmer).

**RNA sequencing and data analysis**

Indexed and purified libraries were loaded together onto
a flow cell, sequencing was carried out on the Illumina
NextSeq 500 platform (paired-end, 2 × 75 bp per read).
Sequencing quality was assessed using FastQC, and Illumina
adapter sequences and low-quality base pairs were removed
using cutadapt version 1.9 [67]. Reads were mapped to the
complete sequenced genome of reference strain EGD-e
(ENSEMBL. ASM19603v1) using Bowtie 2 v2.2.4 with stan-
dard parameters and sensitive-local [68]. BAM alignment files
were used as input for read counting using htseq-count ver-
sion 0.6.0. Differential expression (DE) analyses were per-
formed using DESeq2 in R v3.2.2 [69], and the DE was
reported as log2 fold changes. p-values were adjusted by the
DESeq2 default Benjamini-Hochberg (BH) adjustment
method and genes with a >2-fold (≥1 log2) change in expres-
sion and an adjusted p-value <0.05 were considered as DE.
The transcriptomic data have been deposited in the Sequence
Read Archive (SRA) database and is accessible through the
SRA accession SUB5067488. Volcano plots were drawn by
R software, with the integration of the ggplot2 and ggrepel
graphical packages for data analysis [70]. The top 20 most
significantly upregulated and downregulated expressed genes
in the deletion mutant were labelled. The negative log2 of
p-value was plotted on the Y-axis, and the log2FC was plotted
on the X-axis. The blue points on this graph represent sRNAs
and mRNAs that are significantly differently expressed in the
rlh47 deletion mutant (p-value <0.05); the red points represent sRNAs and mRNAs with p-value >0.05. The horizontal line
represents a p-value >0.05 cutoff. The horizontal lines repre-
sent a −0.58 logFC > 0.58 cutoff. Genes were categorized
into Cluster Orthologous Groups (COGs) and pie charts were
plotted representing the transcript levels of up- and down-
regulated genes grouped by COGs.

**CDNA synthesis and reverse transcriptase-quantitative
polymerase chain reaction (RT-qPCR)**

Fifty micrograms of total RNA were DNase-treated with
TURBO DNA-free kit, according to the manufacturer’s instruc-
tions (Invitrogen™ by Thermo Fisher Scientific). First-strand
cDNA from purified total RNA was synthesized using
SuperScript™ IV First-Strand Synthesis System (Invitrogen™
by Thermo Fisher Scientific) according to the manufacturer’s
instructions. The cDNA quantity was determined by Qubit®
fluorometer (Invitrogen™ by Thermo Fisher Scientific) follow-
ing manufacturer’s recommendations. RT-qPCR was performed
using the Quantitect™ SYBR Green PCR Kit (Qiagen) and
specific primer sets for the genes of interest (Table S1). Primers
for rlh47, ilvA and 16s genes were tested with genomic DNA
prior to analysis. Three sets of cDNA were analyzed, originating
from three sets of RNA extracted as described above. Primers
efficiency was calculated prior to sample runs, in the same
system. The relative expression ratio was used to analyze RT-
qPCR results. The samples were run on a LightCycler® 480
System (Roche) with an initial step at 95°C for 15 min, 45 cycles
of 15 s at 95°C, 15 s at 53°C and 30 s at 72°C, a melting curve
was drawn for 5 s at 95°C, 1 min at 55°C followed by increases of
0.11°C s−1 until 95°C, and a cooling for 30 s at 40°C. Cycle
quantification values were calculated by the software
LightCycler® 480 Software version 1.5.1 (Roche) and the Pfaf
relative expression formula [71,72]. The 16s rDNA gene served
as a reference gene. The experiment was carried out in three
biological replicates, each in technical triplicates.

**Electrophoretic mobility shift assays (EMSAs)**

Templates for *in vitro* transcription carried a T7 RNA poly-
merase binding site at their 5'-end and were generated by
PCR, as described in Lillebeek and Kalliopolitis (2018) [73].
Templates for *in vitro* transcription, RNA purification,
de-phosphorylation and labelling were performed as described
as previously described [65]. Briefly, 40 fmol of 5'-
end-labelled RNA were incubated with a molar excess of
unlabelled RNA in a total volume of 10 μl in the presence
of non-specific competitor (rRNA) for 1 h at 37°C followed by
10 min on ice. Samples were separated on a 5% non-
denaturing gel at 4°C. RNA bands were visualized and ana-
lized as described for the northern blotting experiments.

**Protein extraction and TD colorimetric assay**

Ten µg ml−1 chloramphenicol were added to each of the 25 ml
cell cultures at stationary growth phase. Samples were cen-
trifuged for 15 min at 4°C at 9,000 x g and resuspended in 2 ml
sonication buffer (10 mM Tris-HCl, 0.1 mM EDTA and
5 mM MgCl2, prepared in dH2O and autoclaved, pH 7.4) with
2 mg ml−1 lysozyme (Sigma-Aldrich). Suspensions were incu-

dated at 37°C for 10 min before centrifugation at 9,000
x g for 15 min at 4°C. Pellets were resuspended in 0.3 ml
sonication buffer containing 1% (v/v) protease inhibitor cock-
tail (P2714, Sigma-Aldrich). Cell disruption was performed by
FastPrep-24 (MP Biomedicals). Samples were centrifuged at
13,000 x g for 30 min to remove cell debris. Protein extracts
were quantified by Bradford Protein assay (Bio-Rad).

**Threonine deaminase colorimetric assay**

The activity of TD was assayed using the method of Harris
(1981) [74] with some modifications. Eight hundred µl of
assay buffer consisting of 0.1 M Tris-HCl (pH 8.0),
0.1 M NH4Cl, 0.1 mM pyridoxal phosphate and 50 mM
L-threonine were placed into each 15 ml tube. One hundred
microlitres of cell extract was added to the tubes and incu-
bated in a water bath at 37°C for 20 min. One hundred
microlitres of 50% (v/v) trichloroacetic acid was then added,
followed by 3 ml of 0.025% [w/v] 2,4-dinitrophenylhydrazine
(Sigma-Aldrich) in 0.5 M HCl. After 15 min incubation at 25°C,
1 ml of 10 M NaOH was added. Absorbance at 540 nm was
then measured and recorded for each sample. The amount of
product formed was determined from a standard curve prepared using known concentrations of α-ketobutyrate (Sigma – Aldrich).

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Disclosure of Potential Conflicts of Interest

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References


CHAPTER 5

GENERAL DISCUSSION
Throughout the course of this study, the effect of the Agr and $\sigma^B$ systems in a panoply of environmental conditions that *L. monocytogenes* may encounter was assessed, and a model describing the interactions between these two systems is schematically summarised in **Figure 5.1**. A synergistic effect between the AgrA and $\sigma^B$ regulators was suggested during *L. monocytogenes* survival in the telluric environment and roots colonization. Thereafter, spatiotemporal activation of the Agr and $\sigma^B$ systems was demonstrated during biofilm formation in microfluidic conditions and under osmotic stress, and the impact of one system on the activity of the other was shown. Furthermore, the structure and biological function of the sRNA Rli47 was uncovered for the first time (**Figure 5.1**). This regulatory sRNA specifically represses isoleucine biosynthesis and this activity might contribute to a major regulatory circuitry that acts to restrict growth under harsh conditions.

**Figure 5.1.** Overview of the interactions between Agr and $\sigma^B$ regulators in *L. monocytogenes* (A) adaptation to telluric environments (Chapter 2), (B) in biofilm formation (Chapter 3) and (C) in response to changes in the concentration of BCAA (Chapter 4). Solid arrows indicate activation and crossed lines indicate repression. Solid black lines indicate transcriptional regulation, dotted lines indicate post-transcriptional regulation and dashed black lines indicate an undetermined mechanism of regulation. A red cross indicates gene inactivation.
As we reviewed in the introduction (Chapter 1), while colonising different habitats, *L. monocytogenes* comes across several environmental conditions that are regulated by more than one transcriptional regulator, e.g. virulence (PrfA, CodY, MouR, MogR, AgrA and $\sigma^B$), stress response (CtsR, HrcA and $\sigma$ factors) and biofilm formation (AgrA, $\sigma^B$, MouR). Existing data support the view that the complex, intertwined regulatory network involving numerous transcriptional regulators allows *L. monocytogenes* to fine-tune its response to ever-changing environmental conditions through overlaps among regulons, which are consistent with regulatory redundancies and synergistic control of transcription of particular sets of genes (Chaturongakul *et al.*, 2011; Garmyn *et al.*, 2012; Guariglia-Oropeza *et al.*, 2014; Guldimann *et al.*, 2017; Hu *et al.*, 2007; Liu *et al.*, 2017; Lobel and Herskovits, 2016; Lobel *et al.*, 2012; Milohanic *et al.*, 2003; Ollinger *et al.*, 2009). Global transcription factors that co-regulate multiple pathways simultaneously are essential to this regulatory rewiring. Transcriptional regulators play an important role in the adaptation of bacteria as they allow the modulation of the transcriptome of the bacteria according to the fluctuations in the environmental conditions (Balleza *et al.*, 2009). Furthermore, cooperative behaviours such as virulence, mobility, biofilm formation, antibiotic production or even competence, induced by communication systems allow a better adaptability of bacterial populations (Henke and Bassler, 2004; Hibbing *et al.*, 2010; Keller and Surette, 2006).

An interconnection between the cell-to-cell communication and stress response systems has been described in *S. aureus* (Bischoff *et al.*, 2001). In this pathogen, $\sigma^B$ has a core function in the onset of the bacterial stress response, virulence, biofilm formation, persistence, cell internalization, membrane transport and antibiotic resistance; while the Agr system coordinates the cell-to-cell communication and has been tightly associated with biofilm formation, virulence and adaptation to environmental conditions through the regulation of transcription of a regulatory sRNA, RNAIII, which is the actual effector molecule of this system in *Staphylococcaceae* (Jenul and Horswill, 2018). The regulatory interconnection between the Agr and $\sigma^B$ systems in *S. aureus* is established by repression of RNAIII production by $\sigma^B$ (Bischoff *et al.*, 2001; Lauderdale *et al.*, 2009). High levels of RNAIII are known to have antibiofilm effects suggesting that $\sigma^B$ activity favours *S. aureus* biofilm formation and pathogenesis (Lauderdale *et al.*, 2009; Mitchell *et al.*, 2013; Tuchscherr *et al.*, 2015). Interestingly, another additional level of $\sigma^B$ control is exerted over the Agr system through
increasing of the expression of sarA, which encodes a transcriptional regulator of several virulence factors and activates agr expression (Bischoff et al., 2001; Cheung et al., 2004). A major difference between the agr locus of S. aureus and orthologous systems in several Firmicutes, is that the Listeriaceae family lacks an orthologue of the exclusive staphylococcal effector RNAIII (Wuster and Babu, 2008).

In L. monocytogenes, Garmyn and colleagues (2012) described a temperature-dependent AgrA regulation that overlaps with other regulons, including the σ^B-dependent stress regulon (Garmyn et al., 2012). In fact, σ^B regulates resistance to numerous stresses, aside from occupying a central node in L. monocytogenes regulatory network by interacting with multiple other transcriptional regulators and by regulating the transcription of sRNAs that interfere with gene regulation (Dorey et al., 2019; Mellin and Cossart, 2012). On the other hand, the Agr system has been associated with L. monocytogenes survival in the telluric environment (Vivant et al., 2015; Vivant et al., 2014), in adhesion to abiotic surfaces (Rieu et al., 2007), in the early stages of biofilm formation (Riedel et al., 2009; Rieu et al., 2007; Zetzmann et al., 2019), and host infection (Autret et al., 2003; Riedel et al., 2009). Most recently, Zetzmann and colleagues (2019) have also analysed the overlap between the AgrD and the σ^B regulons of L. monocytogenes, reporting a total of 132 genes (4.6% of genes in the genome) encoding proteins with diverse biological functions, co-regulated by these two transcription factors (Zetzmann et al., 2019). By studying the overlap between the differential transcriptomic data on the ΔagrA at 25°C (Garmyn et al., 2012) with the σ^B stress regulon (Kazmierczak et al., 2003), we identified the four genes of the opuC operon (opuCA-D) within the genes co-regulated by both transcription factors (down-regulated in both ΔagrA and ΔsigB relatively to the wild-type). The genes in the opuC operon encode an ABC transport system responsible for the uptake of carnitine, and were shown to be important for resistance to high salt concentrations (Fraser et al., 2000; Sleator et al., 2001; Wemekamp-Kamphuis et al., 2002). Carnitine is an important osmoprotectant for bacteria, and it is highly present in animal tissues, soil and water (reviewed in Meadows et al., 2015). Altogether, these findings led us to further explore the Agr-σ^B overlap in different contexts of L. monocytogenes lifestyle.

To investigate the contribution of these two systems in response to changing environmental conditions, soil was selected as a model for a complex outdoors habitat integrating abiotic
and biotic parameters. As each soil is a unique habitat characterized by a combination of abiotic and biotic factors (Vivant et al., 2013b), the fate of L. monocytogenes in the telluric environment highly depends on soil composition. L. monocytogenes population has been shown to be stable in clay soils, while it significantly decreases in sandy soils (Dowe et al., 1997; Locatelli et al., 2013a). Moreover, lower soil temperature (25°C rather than 30°C) was also shown to favour L. monocytogenes survival (McLaughlin et al., 2011). Soil moisture is a determinant for survival of L. monocytogenes as desiccation is inhibitory over prolonged periods of time (McLaughlin et al., 2011). Soil pH also seems to influence L. monocytogenes EGD-e persistence, with longer survival periods being reported in soil with pH closer to neutral (32 days at pH 6.5 and 6.9) (Nicholson et al., 2005), whereas shorter persistence was shown for acidic soils (6 days at pH 5.22) (McLaughlin et al., 2011). Soil moisture might affect microbial activity by influencing available oxygen, substrates and water in the soil pore space as well as imposing an osmotic stress. The soil microflora has a significant impact on L. monocytogenes survival as suppression of microflora via soil sterilization permitted better growth of L. monocytogenes than in the presence of a competitive microflora (Dowe et al., 1997; Locatelli et al., 2013b; McLaughlin et al., 2011; Vivant et al., 2013a). Therefore, in this study, we used a clay loamy soil with a pH of 7.15 and an incubation temperature of 25°C. A higher soil water content was shown to favour growth of L. monocytogenes EGD-e populations in sterilized soil. However, changes in the soil’s water content did not cause any differences in survival of L. monocytogenes populations in biotic soil. As expected, a decrease in L. monocytogenes EGD-e populations was noted over time in biotic soil, while growth was observed in sterilized soil (Chapter 2). The inactivation of the soil microbiota was sufficient to generate a favourable habitat for L. monocytogenes to grow as shown by other studies carried out with soils of different types (Dowe et al., 1997; Locatelli et al., 2013b; McLaughlin et al., 2011). Our data confirm that the microflora is the predominant factor in the survival of L. monocytogenes (Chapter 2). Within communities, complex interactions and antibiosis phenomena can develop between microorganisms and regulate microbial communities (Little et al., 2008). Indeed, it was shown before that the inhibitory effect of communities on L. monocytogenes differs according to their phylogenetic composition (Vivant et al., 2013a). The assimilation of carbon sources can also vary according to the species present in the communities, leaving a more or less important access to nutrients for L. monocytogenes. In this regard, some PTS systems are targets of bacteriocins, thus, by blocking the carbon source
transport systems of neighbouring cells, microorganisms could preserve their resources (Kjos et al., 2010). Similarly, the abundance of predators (such as protozoa, bacteriophages, etc.) is likely to modify the impact of communities on the survival of *L. monocytogenes*. Some microorganisms may be more favourable for the survival of *L. monocytogenes* (Gourabathini et al., 2008; Vivant et al., 2013a) – e.g., survival of *L. monocytogenes* populations was shown to benefit from the presence of the amoeba *Tetrahymena pyriformis* (Ly and Muller, 1990). Conversely, the presence of other predatory microorganisms (Olanya and Lakshman, 2015), as well as antimicrobial and bacteriocin-like compounds (Sharma et al., 2014) produced by autochthonous bacteria are inhibitory to *L. monocytogenes*, preventing it from thriving in most outdoor environments. To deepen the knowledge on the possible biotic interactions between telluric microorganisms and *L. monocytogenes*, several approaches can be envisaged. The analysis of the global transcriptome and proteome of the telluric communities would make it possible to evaluate their activities leading to hypotheses on the mechanisms that limit the survival of *L. monocytogenes* in soil.

In our investigations, we sought to determine the effect of the inactivity of both the *Agr* communication system and the stress response regulator $\sigma^B$ on *L. monocytogenes* soil survival (Figure 5.1A). Simultaneous deficiency in both the *Agr* and $\sigma^B$ regulator systems translated in lower fitness of the populations, especially dramatic when both systems were removed. The cell-to-cell communication system has been previously categorised as beneficial to populations of *L. monocytogenes* in the telluric environment. The ability to respond through *Agr* communication provides an advantage to listerial cells to compete with the soil’s endogenous microflora (Vivant et al., 2014). However, when in such a multifactorial environment as soil, *L. monocytogenes* has to cope with ever-changing conditions, and in order to prolong survival the activation of the stress response system seems to be required. During its saprophytic lifestyle, *L. monocytogenes* is likely to encounter several stresses, some of which may occur simultaneously, which might trigger several response mechanisms for bacterial survival and adaptation. Altogether, these phenotypical data suggest that the contribution of the *Agr* and $\sigma^B$ systems seems to impact *L. monocytogenes* soil survival in a synergistic manner (Figure 5.1A), possibly as a result of regulons overlap. In fact, if two genes are co-regulated by the same transcription factor the degree of co-expression may vary (Yu
et al., 2003), suggesting that the existence of regulatory overlaps between these two regulons could result in a synergistic response.

Over recent years, several listeriosis outbreaks were linked to the consumption of contaminated vegetables, e.g. cantaloupe (2011), bean sprouts (2014), lettuce (2015) and frozen vegetables (2016) (CDC, 2018). Apart from these, other leafy greens and ready-to-eat vegetables have been associated with *L. monocytogenes* recalls (de Oliveira et al., 2011). A transfer via direct contact with contaminated soil has been suggested to be the most probable sources of plants contamination by *L. monocytogenes* (Hofmann et al., 2014). However, other sources of contamination by *L. monocytogenes* linked to post-harvest processing cannot be disregarded (Kyere et al., 2018). Hoffman and colleagues (2014) have determined that by introducing a simple washing step with flowing water, the bacterial contamination was either reduced or even removed completely in some cases (Hofmann et al., 2014). However, the findings of Gorski et al. (2011) contradict this by indicating that *L. monocytogenes* attaches so strongly to plant tissues when they are growing in contaminated soil that they cannot be simply washed off (Gorski et al., 2011). Nonetheless, current control steps (washing and radiation) are able to reduce *L. monocytogenes* colonisation to some extent (Kyere et al., 2018). The European Food Safety Authority has a zero-tolerance policy for *L. monocytogenes* in ready-to-eat food throughout the production chain, although the EU-legislation allows 100 CFU/g of *L. monocytogenes* for food already placed on the market, and complete absence of the pathogen in 25 g of sample material before the food has left the operator facilities (EFSA, 2017). Still found in fresh vegetables (Leong et al., 2017; Shenoy et al., 2017), the foodborne pathogen *L. monocytogenes* is able to colonize the plants’ root system, and contaminated vegetables represent a risk for consumers, especially when eaten raw, without thermal processing (Kljujev et al., 2018). However, the fact that *L. monocytogenes* can survive and persist on lettuce leaves and cherry tomatoes in inhibitory conditions (low pH, heat, and osmotic stress), suggests that bacterial cells habituated on fresh produce at low temperatures might acquire resistance to subsequent antimicrobial treatments (Poimenidou et al., 2016).

While the physiology of *L. monocytogenes* in the context of virulence has been studied extensively, the factors that are important for interactions with plants are less well understood. A previous study showed that *L. monocytogenes* could exist on the edges of
damaged seed’s coat during germination, enabling entrance of bacteria into the plant (Gorski et al., 2004). Internalization of L. monocytogenes cells was observed in all major tissue types (the pith, cortex, xylem, phloem, and epidermis), implicating the plant vasculature in the transport of bacterial cells throughout the plant into edible tissues (Shenoy et al., 2017). Moreover, the flagellum represents another important factor of interaction positively affecting L. monocytogenes cell attachment to plant tissues (Gorski et al., 2009). In the present study, we have confirmed the ability of L. monocytogenes EGD-e to colonize the root system of plants when using a F. arundinacea in vitro model (Figure 5.1 A and Chapter 2). No alterations were observed as a result of inactivation of the Agr or σB systems in the initial growth of the population, although the deletion of sigB prevented further growth on the last day of incubation (day 7), suggesting a possible role for σB once the population has established on the root. Impaired attachment and colonisation on radish tissue when σB was inactivated have been reported previously (Gorski et al., 2011). Moreover, our data show that when combined, the inactivation of the two systems (ΔagrAΔsigB) limited L. monocytogenes growth and attachment, with populations decreasing after the first day of inoculation in the roots (Chapter 2). This combined effect of agrA and sigB deletions in L. monocytogenes phenotype might further suggest a synergy between the two systems that may also occur during colonisation by L. monocytogenes of the roots (Figure 5.1 A).

However, it is important to note the presence of unwanted additional point mutations that occurred during construction of the gene knockout mutations in a common genetic background, L. monocytogenes EGD-e. Although complementation of the mutation in trans - i.e. with the relevant wild-type gene on a plasmid (Azizoglu et al., 2014; Chang et al., 2013; Cossart and Mengaud, 1989), is the usual strategy to validate the role of a gene in a phenotype of interest, it can be challenging, if not impossible on some occasions. Several studies reported that σB overexpression in either L. monocytogenes or B. subtilis negatively affects growth or is even lethal (Duncan et al., 1987; Igo et al., 1987; O’Byrne and Karatzas, 2008). It seems that removing sigB from its native genomic context causes problems of deregulation of σB activity, resulting in detrimental sigma factor competition and/or pleiotropic effects on gene expression. For this reason, an alternative strategy was used. Whole-genome sequencing (WGS) was implemented to determine if there were any additional point mutations in a deletion mutants which would remain unknown otherwise, even in the
complemented mutants. WGS provides a rapid and powerful method for screening mutations on a global scale (Smith et al., 2016). Thereafter, we have implemented WGS by Illumina (MicrobesNG, UK) followed by DNA sequence analysis using BreSeq (Barrick et al., 2014) as a routine procedure for screening mutant strains. Through this method we discriminated several single-nucleotide polymorphisms (SNPs) in different genomic locations in the deletion mutant strains, which would have remained unknown otherwise (Chapters 2 and 4). A single SNP was detected in the ΔsigB mutant genome lmo2761 (G1063→T), although this is highly unlikely to have a role in the phenotypic behaviour we described because the SNP detected would produce the change N355Y in the gene product (beta-glucosidase) which, based on an in silico inspection of the protein structure and function prediction, is unlikely to alter its enzymatic function (Peng and Xu, 2011). Two non-synonymous additional point mutations in lmo1255 (G1156→A) and lmo2142 (T527→A) were found in the genome of the Δrli47 mutant. Thereafter, we proceeded to obtain a rli47-complementation strain in order to validate our phenotypic data (Chapter 4). The ΔagrA mutant was previously described (Rieu et al. 2007) and used in several studies (Garmyn et al., 2012; Garmyn et al., 2011; Rieu et al., 2008b; Rieu et al., 2007; Vivant et al., 2015; Vivant et al., 2014). However, in this study we have identified one amino acid changing mutation in this strain relative to the parental, namely in lmo1950 (G369→T), a gene encoding the segregation and condensation protein B. Strikingly, several non-synonymous SNPs were detected in the ΔagrAΔsigB mutant (n=11), namely in rpsR (G8→A), lmo0178 (C964→T), prs (C763→A), ldh (G548→T), sigH (G507→T), lmo0748 (T230→C), lmo1066 (C407→A), lmo1444 (C31→T), lmo2003 (G130→A), lmo2360 (C898→A), and lmo2684 (C962→T). These SNPs were randomly distributed across the genomes and in genes encoding proteins of diverse functions. Selecting clones of the double mutant happened to be difficult and several attempts were necessary. One can hypothesize that construction of the mutant with the extra deletion counter-selected additional mutations counterbalancing fitness problems when both AgrA and σB are inactivated in L. monocytogenes. Similarly, while analysing their L. monocytogenes mutant strain collection lacking distinct LPXTG protein genes, Quereda and collaborators (2013) observed the arising of several mutations, all placed in the sigB operon, which may have resulted from using common genetic procedures or during subculturing (Quereda et al., 2013). This phenomenon has been observed in other bacteria as well, such as Streptomyces lividans, where an additional mutation is required to tolerate the inactivation of RecA, a central enzyme in
genetic instability in this bacterium (Vierling et al., 2001) and E. coli, where additional mutations arose from the inactivation of the Lon protease (Nicoloff and Andersson, 2013). However, in L. monocytogenes, further experiments would be required to support this theory (e.g., repeating the double mutation construction a number of times in different genetic backgrounds to show that this phenomenon always occurs). Alternatively, bad luck could be responsible for picking a colony of a hyper mutator clone during the mutant construction. Overall, WGS should be implemented as standard procedure in the strategy of mutant construction, therefore providing an assertive validation of a gene-dependent phenotype. Further work will be required to confirm definitively if the behaviour of the double mutant is primarily due to the loss of *sigB* and *agrA* or whether it is influenced by one or more of the secondary mutations.

To gather more evidence to support the potential interaction between the Agr and σ^B^ systems suggested by the phenotypic data in soil and roots, we investigated the role of these systems in biofilm formation (Chapter 3). The GFP transcriptional reporter system has been extensively used for applications that require knowledge about the induction of a system activity in response to stress in *L. monocytogenes*, even at the single cell level (Guldimann et al., 2017; Nestler et al., 2009; Utratna et al., 2012). For this study, reporter systems with Agr- and σ^B^-dependent promoters were constructed to assess the spatiotemporal activity of these regulons using GFP as the fluorescent reporter for this purpose (Chapter 3). The overlap between the Agr and the σ^B^ regulons further suggested that these are central nodes in a complex regulatory network that governs the remodelling of the transcriptome during biofilm formation (Zetzmann et al., 2019), therefore, biofilm formation was selected as the preferential condition to reassess this interaction.

As a mechanism of increasing bacterial persistence and survival, the foodborne pathogen *L. monocytogenes* forms resilient biofilms over a wide range of temperatures from 4 to 42°C, which are especially problematic in food-producing industries (Di Bonaventura et al., 2008; Tomićič et al., 2016). Once in the food-processing environment, sessile *L. monocytogenes* populations are able to survive and persist under food-related conditions such as exposure to disinfectants, cold temperatures, desiccation, heat, high salt content and shear stresses (Bucur et al., 2018; Doyle et al., 2001; Ferreira et al., 2014; Gardan et al., 2003; Rodriguez-
Lopez et al., 2018; Tasara and Stephan, 2006; Zoz et al., 2017). From another perspective, L. monocytogenes may as well experience some of these adverse conditions when within the host. As a foodborne and facultative intracellular pathogen, L. monocytogenes faces shear stresses in both food-related and host reservoirs. In the food-processing environment L. monocytogenes faces shear stresses when in drains and pipes (Di Ciccio et al., 2012; Liu et al., 2016) while, once in the host, shear forces could be found in the gastrointestinal tract and in the blood circulation in the latter stages of an infection (Davies, 1995; Hansson, 2012; Soyer and Dumenil, 2011). Nonetheless, biofilm formation increases survival, even when bacteria encounter hydrodynamic conditions. The microfluidic system BioFlux has been used to study the biofilm formation of several bacterial species (Diaz De Rienzo et al., 2016), and recently this device was used to study L. monocytogenes biofilm formation for the first time (Cherifi et al., 2017). Coupled to fluorescence microscopy, the BioFlux system enables the assessment of real-time biofilm formation under microfluidic growth conditions (Meyer et al., 2011), while accurately controlling temperature and flow rates, as well as permitting simultaneous growth of multiple biofilms (Benoit et al., 2010), which made of this the ideal tool for obtaining data from our collection of reporter strains during biofilm formation and in response to osmotic stress.

In nature, bacteria can alternate between two modes of growth as planktonic cells free-swimming in aqueous environments or as a sessile biofilm community. When transitioning from planktonic growth to biofilm formation, bacteria activate complex regulatory network of different signalling pathways. Among others, nucleotide second messengers such as cyclic di-GMP (c-di-GMP), quorum sensing molecules, sigma factors and two-component regulatory systems play an important role in biofilm development (Lopez et al., 2010; Valentini and Filloux, 2016). In Gram-negative bacteria species, e.g. E. coli, Pseudomonas aeruginosa, and S. enterica serovar Typhimurium, the signalling molecule c-di-GMP coordinates the switch from planktonic to sessile growth as the cells perceive intracellular accumulation of this molecule as a signal for stimulating biofilm lifestyle (Simm et al., 2004), despite playing an important role in mediating stress response in microbial communities (Chua et al., 2015). However, in Gram-positive bacteria, other molecules can drive bacterial cells to associate in a biofilm; in quorum-sensing systems secreted peptides are used as signal molecules, which are detected by two-component regulatory systems that then trigger changes in gene
expression (Kleerebezem et al., 1997). The Agr system is included in the bulk of these communication systems which not only mediates regulation of biofilm behaviour but also virulence (Garmyn et al., 2009). Several studies have reported the involvement of the Agr system in cell attachment to the surface (Riedel et al., 2009; Rieu et al., 2008a; Rieu et al., 2007; Zetzmann et al., 2019). However, this communication system does not seem to play any role in the biofilm structure, which further implies that the requirement of this system might occur only during early stages of biofilm formation. Indeed, our expression data suggested that Agr activation was kept constant throughout biofilm formation, although an increase in fluorescence was shown through time (Chapter 3). These led us to conclude that during biofilm development, the Agr-dependent regulation may be transitory. After adhesion, *L. monocytogenes* might undergo major gene expression alterations which may further require Agr activity.

Apparently in disagreement with previous investigations (van der Veen and Abee, 2010b), our data showed that the inactivation of the stress response regulator $\sigma^B$ resulted in an increment of static biofilm production in rich medium (Chapter 3). The culture conditions such as temperature, medium and methods used in these different studies could be the reason for the discrepancies observed. Although $\sigma^B$ is not essential for cell attachment to the surface (Schwab et al., 2005), the absence of $\sigma^B$ hampered cell attachment to the surface. However, a subsequent decrease in biofilm development by the $\Delta sigB$ mutant was observed under osmotic stress in microfluidic conditions. The loss of each regulator resulted in changes in *L. monocytogenes* biofilm development; reduced adherence on early biofilm formation on static abiotic surfaces in the $\Delta agrA$ mutant was described (Rieu et al., 2007), as well as significant decrease in the amount of biofilm formed by a $\Delta sigB$ mutant (van der Veen and Abee, 2010b). Reported in previous studies (Garmyn et al., 2012; Zetzmann et al., 2019), the differential expression of a remarkably large number of genes connected to stress response and resistance to atypical conditions when the Agr system in not functional points out to overlaps between the Agr and $\sigma^B$ systems in *L. monocytogenes*. Indeed, the deletion of *agrA* or *sigB* altered the activation of the $\sigma^B$ and Agr systems, respectively (Figure 5.1 B). Activation of Agr is kept constant during biofilm formation in the absence of $\sigma^B$. Moreover, the loss of $\sigma^B$ accentuated the inactivation of Agr-dependent promoters in biofilm formed in the presence
of salt stress. Conversely, earlier activation of $\sigma^B$ in biofilm occurs when the communication system Agr is inactivated, even when under osmotic stress.

A heterogeneous activation of Agr and $\sigma^B$ was observed in our expression data (Chapter 3). Despite the numerous benefits that a bacterial community obtains from biofilm formation (e.g., resistance to antimicrobials, and protection from protozoa predation and host’s defence mechanisms) (Anderson and O’Toole, 2008; Mah and O’Toole, 2001; Matz and Kjelleberg, 2005), this structural formation may also account for some downsides such as promoting intense competition for space and hampering the diffusion of nutrients, vitamins and cofactors across cells in the populations, which inherently results in some metabolically inactive micro-niches within the bacterial community and consequently cell competition for these resources (Stewart and Franklin, 2008). Depletion of resources in high cell densities in biofilms results in progressive deterioration of conditions, a situation that is boosted by the accumulation of metabolic wastes and culminate in either cell inactivity and ultimately death (Serra and Hengge, 2014). Therefore, cells might sense different environmental cues throughout the biofilm, hence activating different stress responses, despite fitting the bet-hedging theory (Veening et al., 2008a; Veening et al., 2008b). Within a bacterial population exposed to an environmental condition, gene expression in cells is inherently noisy, and often shows stochastic patterns (Kaern et al., 2005; Raj and van Oudenaarden, 2008). Despite bet-hedging, where the individual cells expressing the required molecules will have a relatively increased chance of surviving future sudden environmental changes, stochastic differences in gene expression can also be explained by labour-sharing, where only a few cells from specialized subpopulations carry the burden of producing a molecule that will eventually benefit the entire population (Eldar and Elowitz, 2010). While providing insights on PrfA and $\sigma^B$ activation in L. monocytogenes under stress conditions, Guldimann et al. (2017) perceived that these systems are differently activated within a population. If on the one hand, having a large number of cells with active PrfA in a population under certain conditions seems advantageous; on the other hand, $\sigma^B$ activity is restricted to a relatively small number of cells as what appears to be the result of a labour-sharing and/or bet-hedging mechanism (Guldimann et al., 2017). While the constitutive activation of PrfA (PrfA*) is viable (Ripio et al., 1997), activity of $\sigma^B$ comes at such cost to the cells of L. monocytogenes (Chaturongakul
and Boor, 2004) that its overexpression results in poor growth if not with a deleterious effect (Cosgrave, 2010).

Salt stress also seemed to influence the activity of both AgrA and σB regulators, as a clear role for σB in osmotic tolerance was identified, while a decrease in agrA transcription was promoted by low NaCl content (Abram et al., 2008a; Larsen and Jespersen, 2015). L. monocytogenes experiences osmotic stress not only in the host gastrointestinal tract (Barbosa et al., 2012), but also in food as the result of increased concentrations of salts or sugars that are added to improve the sensory properties and as preserving agents to increase the shelf life (Burgess et al., 2016). Our results demonstrated that the presence of osmotic stress reduces L. monocytogenes biofilm formation (Figure 5.1 B) (Chapter 3). No major activation of Agr was observed throughout biofilm formation under osmotic stress, undermining a direct role of the Agr regulon in response to these conditions. The role of σB in limiting biofilm development was also observed in biofilm formed under osmotic stress. In L. monocytogenes, σB has been shown to decrease growth rate in response to osmotic stress, and an increase in growth is caused by the inactivation of σB (Abram et al., 2008a). Nonetheless, even though the stress response factor σB is strongly active in stationary phase cells or in cultures supplemented with NaCl (Utratna et al., 2011), in biofilm formed under the same circumstances σB activity was generally low. This suggests that something is triggered during biofilm formation that reduces σB activity even under osmotic stress, or simply that since diffusion distance is increased in the biofilm mode of growth relatively to growth in planktonic cultures (Stewart, 2003), not all cell subpopulations are expected to be equally exposed to the same amount of stress, causing differences in activation of the general stress response.

While establishing biofilms, L. monocytogenes might integrate multiple regulatory inputs simultaneously. During sessile growth, Agr activity depended on the stage of biofilm development, suggesting that this system is important during early stages of biofilm formation (Figure 5.1 B). In S. aureus and S. epidermidis, agr-dependent regulation of the expression of several adhesion proteins has been demonstrated (Cramton et al., 1999; Mack et al., 2007). This has not been shown in L. monocytogenes, although it suggests that the Agr system could also regulate the expression of proteins necessary for the ability to attach
to abiotic surfaces without being involved once the cells attach to the surface. At the moment, not much is known about the requirement of stress response genes in *L. monocytogenes* biofilm development. σ^B activity does not seem to be essential for initial attachment and sessile growth (Schwab et al., 2005), although it is required later on in the process of biofilm formation (van der Veen and Abee, 2010b). A role in biofilm development was attributed to σ^B in *S. aureus* and *S. epidermidis*, through the regulation of the ica operon, encoding the polysaccharide intercellular adhesin involved in the formation of *Staphylococcus* biofilms (Argudin et al., 2015; Knobloch et al., 2004; Rachid et al., 2000). In nature, however, bacteria rarely have unlimited nutrients and very frequently encounter hostile factors in the form of host immune system or environmental stress. Thus, the biofilm mode of growth results in micro-niches in which bacteria experience stress and therefore activate various stress resistance mechanisms in response to hostile factors (Costerton et al., 1995). When considering that while attaching to a surface, cells have less options for responding to stress, a survival strategy might pass by triggering σ^B activity at this early stage preparing cells for hypothetical outcome stressful events (Costerton et al., 1995). Therefore, coupling stress sensing with mechanisms to sense population densities appears to be a likely strategy adopted by *L. monocytogenes* in biofilm behaviour. The inactivation of both AgrA and σ^B regulators compromises adaptation to studied conditions, suggesting that cooperation of these two regulators for *L. monocytogenes* survival in the outdoors environment may be important (Figure 5.1 A). The hypothesis of an overlap between the AgrA and σ^B regulons arose as variations in the expression of these promoters resulted from the absence of the regulator of the other system during continuous flow-biofilm production.

Taken together our results from these studies seem to suggest a redundancy in the regulation of these two key survival mechanisms, where a synergistic effect might outcome from simultaneous activation of both regulators, and the loss of one can be compensated for by the other (Chapters 2 and 3). More research needs to be carried out in order to investigate the nature of this interconnection. For instance, it would be interesting to evaluate the dynamics of *L. monocytogenes* as planktonic cells and in a biofilm community through transcriptomic analyses, identifying the Agr and σ^B co-regulated genes at play in the biofilm circuitry under different environmental conditions. It could help identifying the biological reason for this regulatory redundancy. At the single cell level, a double reporter system would
permit the activation of Agr and $\sigma^B$ to be followed through time, providing a simultaneous insight on the activation of these regulators in a population over time.

Aiming to unveil the molecular mechanisms involved in the interconnection of AgrA and $\sigma^B$ regulators, while knowing that in S. aureus these two interact through a regulatory sRNA (Bischoff et al., 2001), we searched the bibliography for reports of sRNAs directly or indirectly under the control of both regulators. The sRNA Rli47 was identified in the intersection of both AgrA and $\sigma^B$ regulons (Figure 5.1 A and C). This sRNA was shown highly $\sigma^B$-dependent and also overexpressed in the $\Delta$agrA mutant relative to the parental strain L. monocytogenes EGD-e during soil adaptation (Vivant et al., 2015). Possibly due to the different experimental setup, we did not observe significant differences in Rli47 transcript levels between the $\Delta$agrA mutant and the parental strain from our soil experiments (Chapter 2). However, we further showed a relative increase in Rli47 expression in the $\Delta$agrA strain under standard lab conditions. Moreover, we further showed a strong $\sigma^B$ dependance of Rli47, with expression of Rli47 being completely abolished in the $\Delta$sigB mutant (Chapter 4). Therefore, Rli47 was initially studied as part of this thesis in the context of providing more evidences of the interconnection between AgrA and $\sigma^B$ in L. monocytogenes. Mapping Rli47 in the genome of L. monocytogenes EGD-e important, given the considerable amount of inconsistent information regarding the size of this sRNA. Several studies suggested a biological role of Rli47 in the virulence process (Mraheil et al., 2011; Toledo-Arana et al., 2009) and in response to oxidative stress (Mujahid et al., 2012), although it remained inconclusive. However, in the chapter 4, we uncovered a biological role of Rli47 for the first time.

Our study showed that Rli47 is responsible for specifically repressing isoleucine biosynthesis through the repression of translation by occlusion of the predicted ilvA ribosome binding site, and probably by affecting the mRNA stability as a way to restrict growth under harsh conditions (Chapter 4). These results clearly showed that Rli47 acts to repress BCAA biosynthesis. Although the abundancy of Ile in the gastrointestinal tract of the host is unquantified, Brenner et al. (2018) have suggested that L. monocytogenes evolved a controlled BCAA auxotrophy mechanism, enabling Ile to serve as a host signal and virulence effector (Brenner et al., 2018). Additionally, this study has identified another layer of the complex regulation of the ilv-leu operon in L. monocytogenes, which is accomplished by the
combined actions of CodY and the ribosome-dependent transcription attenuator Rli60 (Brenner et al., 2018).

A link between metabolism and virulence gene expression is made by CodY which positively regulates PrfA expression in response to low BCAA levels (Brenner et al., 2018; Lobel et al., 2012; Toledo-Arana et al., 2009). In addition to controlling rli47 and prfA transcription, our results suggest that σ^B acts as a repressor of BCAA biosynthesis pathway by decreasing threonine deaminase activity and impairing adaptation to Ile starvation. Therefore, the general stress response regulator σ^B might as well link BCAA and virulence by acting via another unknown mechanism (Figure 5.1 C). It is possible that stresses encountered within the host, particularly in the gastrointestinal tract where σ^B is known to be active (Toledo-Arana et al., 2009), trigger this additional layer of negative control on BCAA biosynthesis. Considering all these hypotheses, the binding of Rli47 to ilvA mRNA is expected to occur as an additional layer providing another level of negative control that operates to restrict isoleucine biosynthesis even when this amino acid is absent from the growth medium. Despite encoding all the BCAA biosynthesis genes, L. monocytogenes still requires BCAA supplement to support growth (Joseph and Goebel, 2007; Premaratne et al., 1991). Although more evidence is required to support the theory that Ile acts as a host-specific signal, the Rli47-mediated effect could serve as an early stimulus to prime cells for virulence by inducing prfA transcription in response to σ^B activation. Further studies could help to deeper explore this idea (e.g., gene expression and phenotype experiments could provide an insight into the effect of the absence of Rli47 in prfA transcription and activation throughout L. monocytogenes virulence process).

Despite being studied intensively, the function of many regulatory sRNAs in L. monocytogenes remains elusive (Becavin et al., 2017; Mellin and Cossart, 2012; Thorsing et al., 2018). The most used work-flow for determining the role of a regulatory sRNA relies on in silico predictions followed by in vitro confirmation and subsequent validation in vivo (Dos Santos et al., 2018; Mollerup et al., 2016; Quereda et al., 2014; Sievers et al., 2014). However, a regulatory sRNA might not target a single mRNA in the cell. Different sRNAs can target multiple mRNAs, and a single mRNA can be targeted by multiple regulatory sRNAs, e.g. the Rli22 and Rli33-1, members of the LhrC family of sRNAs in L. monocytogenes both target multiple mRNAs, which they share among the two as well as with the other five LhrC-family
members (LhrC1-5) (reviewed in Thorsing et al., 2017). With this in mind, we used a differential RNA-seq analysis as a supplementary approach to the classical in silico prediction for determining hypothetical targets of Rli47 in L. monocytogenes (Chapter 4). Transcription of an estimated total of 155 genes was shown to be affected by Rli47, most of which are involved in amino acid transport and metabolism, and several overlaps with other transcriptional regulators (AgrA, $\sigma^8$, CodY and PrfA) were observed. RNA-seq analysis suggested further direct or indirect regulatory roles of Rli47 in cobalamin (vitamin B12) biosynthesis. Cobalamin is one of the most structurally complex small molecules made in nature, which production is confined to a few microorganisms (Martens et al., 2002). There are two different pathways for cobalamin biosynthesis in nature, a pathway dependent on oxygen, and an oxygen-independent, anaerobic pathway. Genes encoding enzymes contributing to the oxygen-dependent cobalamin biosynthesis are recognized by the prefix cob, while genes involved in the oxygen-independent pathway are usually named using the prefix cbi (Martens et al., 2002). The absence of Rli47 generated a decrease in the transcript levels of copB, a gene encoding adenosylcobinamide-phosphate guanylyltransferase, a bifunctional cobalamin biosynthesis protein. Interestingly, the copB mRNA was one of the in silico predicted Rli47 targets but in vitro binding assays failed to demonstrate their interaction. However, it could be that the in vitro binding conditions used for EMSA analysis failed to emulate the conditions within the cytoplasm in some crucial respect. Interestingly, the transcription of another gene involved in cobalamin biosynthesis, cbiD (cobalt percorrin-6A synthase), was found equally up-regulated by Rli47. In this context, Rli47 might contribute to an increase in cobalamin biosynthesis in L. monocytogenes under specific micro-niches. Several genes associated with energy production and conversion, including pyruvate metabolism, were found differentially expressed in the absence of Rli47, such as the genes encoding the bifunctional acetaldehyde-CoA/alcohol dehydrogenase (lmo1634), pyruvate-formate lyases (pflA, pflC and pflB), and succinate dehydrogenase (lmo0355). This highlights a hypothetical contribution of Rli47 in the shifting of the energetic status of the cell under specific conditions. Furthermore, this study suggested multiple possible regulatory roles of Rli47, considering its effect on changing the transcript levels of genes involved in nucleotide biosynthesis (purH, purN and purM), putative transcriptional regulators (lmo2365, lmo2364 and lmo2447), pheromones (lmo2637), late competence (lmo1341), secretion (esaC), transferases (lmo1201) lipid transport and metabolism (yjil, iolI) and transport systems (feoA, feoB, feoC).
feoB Imo2497, Imo0861 and Imo0135). This data may provide a lead to future investigations on establishing the involvement of Rli47 in the expression of these genes, as well as determine the molecular mechanisms behind these.
CHAPTER 6

CONCLUDING REMARKS
Over the years, fundamental research on *L. monocytogenes* ecology, molecular genetics and physiology has greatly improved our understanding of the biology of this pathogen, although it remains a major concern to food industries and human health. While transitioning from different habitats, this highly adaptable microorganism avails of different molecular mechanisms to sense its surroundings and quickly orchestrate a response that ultimately leads it to thrive under the most challenging conditions. In conclusion, in this study we have shown that the Agr communication system and the stress response regulator $\sigma^B$ are involved in a complex regulatory network and play a crucial role in the adaptation, survival and competitiveness of *L. monocytogenes*. However, the nature of the regulatory links between Agr and $\sigma^B$ in *L. monocytogenes* remains to be fully clarified. As demonstrated in this thesis several lines of evidence suggest that these two systems interact in previously unforeseen ways during *L. monocytogenes* saprophytic lifestyle, either in soil, the rhizosphere or during biofilm formation. While looking for potential mechanisms linking the AgrA and $\sigma^B$ regulons, we identified Rli47, a small regulatory RNA whose biological function we were able to partially determine for the first time. By binding to the predicted regulatory region of ilvA mRNA, the sRNA Rli47 is responsible for specifically repressing isoleucine biosynthesis, restricting growth under harsh conditions as a way to contribute to the survival of *L. monocytogenes*. Furthermore, the present study suggests a list of other hypothetical targets for Rli47, providing leads for further investigations to more deeply explore the regulatory role of this sRNA.

While a number of novel findings were made, many more interesting questions remain in these areas of research. The importance of this bacterium as a foodborne pathogen will ensure that it continues to be studied extensively for the foreseeable future. There are still significant gaps in our knowledge, one of which concerns the precise mechanisms that *L. monocytogenes* uses to sense its environment and to defend itself against harsh conditions. With regards to further advancing the knowledge of regulatory networks on *L. monocytogenes*, gathering more information of the precise biological function of a network and its stakeholders poses as one of the current challenges. However, research in these fields is likely to produce answers to these questions in the near future. A detailed knowledge of the molecular mechanisms that this pathogen requires to transition between different environments might ultimately facilitate the development of better strategies to help food
producers to combat *L. monocytogenes* contamination within their facilities, as well as prompting biotechnological applications to help prevent listeriosis.


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facilities form more biofilm but are not linked to specific genetic markers. Int J Food Microbiol 256, 45-53.


Occurrence of mutations impairing sigma factor B (SigB) function upon inactivation of *Listeria monocytogenes* genes encoding surface proteins. Microbiol 159, 1328-1339.


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- Ao Tico, por seres o melhor irmão do Mundo. Pelas visitas, pelas mensagens, pelos telefonemas, por me acompanhares também nas adversidades e por me encheres de orgulho todos os dias.

Obrigada.
SUPPLEMENTARY MATERIAL

Chapter 4. The $\sigma^B$-dependent regulatory sRNA Rli47 represses isoleucine biosynthesis in Listeria monocytogenes through a direct interaction with the ilvA transcript.

![Diagram](image)

**Figure S1.** Mapping the transcription start site of *rli47*. (A) Illustration of the genomic location and promoter region sequence for *rli47*. Transcription start site (+1) is marked by an arrow. The -35 and -10 boxes are indicated. (B) Comparison of Rli47 levels in ΔsigB, ΔagrA, and BHI LB+Glucose control strains. (C) Gel electrophoresis showing the transcription start site of *rli47* in ΔsigB, ΔagrA, and BHI LB+Glucose control strains. 1,500 nt.
10 boxes of the σ^8 promoter (P_8) upstream rli47 are marked in bold. The lollipop structure denotes a hypothetical transcription terminator structure. (B) The 3’-end of rli47 was postulated considering the size reported in Wurtzel and collaborators (2012) and a hypothetical transcription terminator structure overlapping lmo2142. Different probes for northern blots (see Table S1) flanking this region determined that the terminator structure was part of Rli47. (C) Primer extension analysis was performed to determine the 5’-end of rli47 on stationary cells in BHI and LB + Glucose. Hypothetical transcription terminator is shown underlined. The transcription start site (+1) is marked in bold and the -10 box is indicated.

Figure S2. Original northern blot analysis on Rli47 transcription in cells harvested at exponential and stationary phases in TSB and DM. Samples were taken from L. monocytogenes EGD-e wild-type (WT), ΔsigB and Δrli47 cultures at exponential and stationary phases in TSB and DM at 37°C, and loaded in a gel per growth stage. Northern blot was probed for Rli47 and 16S rRNA as a loading control. Representative of two independent technical replicates of two biological samples per condition.
Figure S3. Northern blot analysis on Rli47 transcription on a σ8-dependent manner. Samples were taken from *L. monocytogenes* EGD-e wild-type (WT), ΔsigB and Δrli47 cultures at exponential and stationary phases in TSB at 37°C. Northern blot was probed for Rli47 and 16S rRNA as a loading control. Relative levels of Rli47 were normalized to 16S and are shown below each lane. Representative of two independent technical replicates of two biological samples per condition.

Figure S4. Impact of the deletion in rli47 on the transcription of the flanking genes *lm02141* and *lm02142*. Quantification of *lm02141* and *lm02142* transcript levels was assessed in the Δrli47 relative to the wild-type by RT-qPCR and normalized to 16S rRNA levels. Logarithmic fold change values on the mutant strain are relative to the wild-type (wt/mutant) for cells harvested in stationary phase in TSB and exponential phase in DM. The data represent three biological replicates with three technical independent repetitions. Error bars indicate standard deviation. The *p*-value was calculated using a Student *t*-test with a Welch’s corrections (* = *p*-value <0.05; ns – not significant).
Figure S5. Mutational Analysis of truncated Rli47 and ilvA mRNA base pairing by Electrophoretic Mobility Shift Assay. Labeled sRli47 and sRli47GGUG were each incubated with increasing concentrations of unlabeled ilvA RNA or the mutant ilvA-SD\textsuperscript{ACC}. The fraction of unbound Rli47 is shown below each lane.

Figure S6. Impact of sigB or rli47 deletion on ilvA expression in stationary growth phase cells in TSB. Quantification of rli47 and ilvA transcript levels was accessed in the Δrli47 and ΔsigB relative to the wild-type by RT-qPCR and normalized to 16S. Logarithmic fold change values on the mutant strains are relative to the wild-type. The data represents three biological replicates with three technical independent repetitions. Error bars indicate standard deviation. Asterisks represent p-values (\(* = p-\)
value <0.05, ** = p-value <0.001, *** = p-value <0.0001) calculated using a Two-way ANOVA with Sidak’s multiple comparisons test.

Figure S7. Assessing the effect of Rli47 on the branched-chain amino acids isoleucine pathway under isoleucine depletion. Growth of L. monocytogenes EGD-e wild-type (WT), ΔsigB and Δrli47 in chemically defined medium (DM) containing a 0, 8, 80 and 800 μM L-isoleucine, supplemented with 800 μM L-threonine. Bacterial growth at 37°C was monitored until all cultures reached stationary phase. The average of three biological replicates is shown and error bars represent standard deviation. Doubling and lag times are shown on Table S3.
### Table S1. Primers and probes used in this study.

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<th>Sequence (5’ → 3’)</th>
<th>Further information</th>
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<td>rli47_F</td>
<td>TGGCTTTAAACAGGAAGGCT</td>
<td>Forward primer for upstream flanking region of rli47.</td>
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<tr>
<td>rli47_R</td>
<td>ACTAAGCCAGACACTGCAGA</td>
<td>Reverse primer for downstream flanking region of rli47.</td>
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<td>sigB_F</td>
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<td>Northern blotting probe for Rli47.</td>
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<td>Rli47(3)_NB</td>
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**EMSA**s

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<td>Forward primer, synthesis of rli47 DNA with T7 promoter to be transcribed into RNA.</td>
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<td>CM_5_R</td>
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<td>Forward primer for synthesis of rli47 truncated version.</td>
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<td>GGGGTAAATACGACTCATATAGGGCAAACGAAATAAAGGTGA</td>
<td>Forward primer for synthesis of copB DNA with T7 promoter to be transcribed into RNA.</td>
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<tr>
<td>CM_20_R</td>
<td>CCCGCCAGTATCATCAGCAT</td>
<td>Reverse primer for synthesis of copB.</td>
</tr>
<tr>
<td>CM_22_R</td>
<td>GAATGATACGATCTATTCGCTTAATGATGTCACCATTTATGACGCATCCAATAAATTATGACGCATCAATG</td>
<td>Reverse primer for synthesis of rli47 truncated version with mutated loop sequence to be transcribed into RNA (sRli47(s)).</td>
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<tr>
<td>CM_23_R</td>
<td>TTCTGTATTTAATGAACTGCGT</td>
<td>Reverse primer for synthesis of ilvA DNA with mutated SD sequence to be transcribed into RNA (ilvA-SD(s)).</td>
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</table>
Table S2. Plasmids and strains used in this study.

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<th>Plasmids</th>
<th>Genotype and further information</th>
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</tr>
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<tr>
<td>pCM001</td>
<td>pMAT_Δrli47, insert of 552 bp spanning the upstream and downstream flanking regions of rli47 with a Δ440 bp (19_458del)</td>
<td>GeneArt, ThermoFisher Scientific</td>
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<tr>
<td>pCM002</td>
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<tr>
<td>pCM003</td>
<td>pMK4_rli47, insert including rli47 gene with its own promoter region (-91C_536T)</td>
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<table>
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<th>Strains</th>
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<td>WT</td>
<td>Listeria monocytogenes EGD-e</td>
<td>Glaser et al., 2001</td>
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<tr>
<td>DG125A</td>
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<td>Rieu et al., 2007</td>
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<td>CM007</td>
<td>Listeria monocytogenes EGD-e ΔsigB</td>
<td>In this study</td>
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<td>CM009</td>
<td>Listeria monocytogenes EGD-e Δrli47</td>
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</tr>
<tr>
<td>CM016</td>
<td>Listeria monocytogenes EGD-e Δrli47, pMK4::rli47</td>
<td>In this study</td>
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<tr>
<td>E. coli TOP10</td>
<td>E. coli TOP10</td>
<td>Invitrogen</td>
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Table S3. Growth rates of WT and mutant strains grown in chemically defined media differently supplemented with threonine and isoleucine. Lag and Doubling times were calculated by GrowthRates 3.0. The data represents three biological replicates with three technical independent repetitions. Error bars indicate standard deviation. Asterisks represent p-values (* = p-value <0.05, ** = p-value <0.001, *** = p-value <0.0001) calculated using a Two-way ANOVA with Sidak’s multiple comparisons test.

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<th>Medium</th>
<th>Strain</th>
<th>Lag time (min)</th>
<th>Doubling time (min)</th>
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<td>DM (800 μM Ile)</td>
<td>WT</td>
<td>98.37 ± 6.19</td>
<td>139.70 ± 14.98</td>
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<tr>
<td></td>
<td>ΔsigB</td>
<td>92.73 ± 8.09</td>
<td>126.10 ± 3.41</td>
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<tr>
<td></td>
<td>rli47-c</td>
<td>100.47 ± 1.81</td>
<td>154.83 ± 1.81</td>
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<tr>
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<td>Δrli47</td>
<td>87.27 ± 2.54</td>
<td>129.40 ± 8.29</td>
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<tr>
<td>DM - Ile</td>
<td>WT</td>
<td>1,330.53 ± 46.6</td>
<td>109.93 ± 4.54</td>
</tr>
<tr>
<td></td>
<td>ΔsigB</td>
<td>550.8 ± 121.84 *</td>
<td>113.07 ± 9.83</td>
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<tr>
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<td>rli47-c</td>
<td>1,959.3 ± 60.58 ***</td>
<td>101.17 ± 1.10</td>
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<tr>
<td></td>
<td>Δrli47</td>
<td>1,050.3 ± 67.73 ***</td>
<td>114.3 ± 21.86</td>
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<tr>
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<td>WT</td>
<td>93.27 ± 2.80</td>
<td>141.03 ± 7.06</td>
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<tr>
<td></td>
<td>ΔsigB</td>
<td>87.13 ± 0.50</td>
<td>106.63 ± 13.73 **</td>
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<tr>
<td></td>
<td>Δrli47</td>
<td>89.63 ± 2.22</td>
<td>143.27 ± 5.90</td>
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<tr>
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<td>184.27 ± 1.92 ***</td>
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<td>Δrli47</td>
<td>977.97 ± 20.57 ***</td>
<td>147.5 ± 4.10 **</td>
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<td>ΔsigB</td>
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<td>142.60 ± 9.61 *</td>
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<td>1,293.77 ± 7.43 ***</td>
<td>99.47 ± 6.04 *</td>
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Table S4. Results of RNA-seq data for Δrli47 vs wild-type in exponential phase cells grown in DM. p-value > 0.05 and -1.5< Fold-change >1.5 cutoffs were applied.

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<td>Not in COGs</td>
<td>Not in COGs</td>
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<td>hypothetical protein - RAST_Product: Pyruvate formate-lyase (EC 2.3.1.54) similar to pyruvate formate-lyase</td>
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**Upregulated genes in Δrli47 vs wild-type**
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<th>p-value</th>
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<td>Q8Y5K2</td>
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<td>Amino acid transport and metabolism</td>
<td>Operon_381</td>
<td>argininosuccinate synthase similar to argininosuccinate synthase catalyzes the formation of 2-N-(omega-L-argininyl)-L-argininosuccinate from L-citrulline and L-aspartate in arginine biosynthesis, AMP-forming</td>
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<td>argininosuccinate lyase activity [GO:0004056]</td>
<td>Amino acid transport and metabolism</td>
<td>Operon_381</td>
<td>argininosuccinate lyase similar to argininosuccinate lyase catalyzes the formation of arginine from (N-L-arginino)succinate</td>
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<td>Acetamidohydrolase activity [GO:0042802]; N-acetyl-L-ornithine-2-oxoglutarate S-amino transferase activity [GO:0003992]; pyridoxal phosphate binding [GO:0030170]</td>
<td>Amino acid transport and metabolism</td>
<td>Operon_270</td>
<td>acetamidohydrolase amine transferase highly similar to N-acetamidohydrolase amine transferase</td>
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<td>Amino acid transport and metabolism</td>
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<td>Operon_O84</td>
<td>shikimate 5-dehydrogenase similar to shikimate 5-dehydrogenase AroE catalyzes the conversion of shikimate to 5-dehydroshikimate.</td>
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<td>Posttranslational modification, protein turnover, chaperones</td>
<td>Operon_O84</td>
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<td>3-deoxy-D-arabino-heptulosonate 7-phosphate synthase</td>
<td>Alddehyde lyase activity [GO:0015632]; metal ion binding [GO:0046872]; transferase activity [GO:0016740] Amino acid transport and metabolism</td>
<td>Operon_O84</td>
<td>bifunctional 3-deoxy-7-phosphoketolase synthase/chorismate mutase catalysts the formation of 3-deoxy-D-arabino-hept-2-ulosonate 7-phosphate from phosphoenolpyruvate and D-erythrose 4-phosphate and the formation of prephenate from chorismate.</td>
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Upregulated genes in Δrli47 vs wild type
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<td>Indolylglycerol phosphate dehydratase activity [GO:0006424]</td>
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<td>Indolylglycerol-phosphate dehydratase catalyzes the dehydration of D-erythro-1-imidazol-4-ylglycerol 3-phosphate to L- (imidazol-4-yl)-3-o-oxopyrophosphate in histidine biosynthesis</td>
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**CAC98603**
Gene: lmo0725 | Loci: lmo0725 | p-value: 0.01165157210640 | Log2FC: 0.65 | FC: 1.6 | Entry: Q8P917 | Protein names: Putative peptidoglycan bound protein (LPXTG motif) | Gene ontology: Not in COGs | COG: peptidoglycan binding protein |

**CAC99814**
Gene: lmo1736 | Loci: lmo1736 | p-value: 0.00743737138511 | Log2FC: 0.64 | FC: 1.6 | Entry: Q8Y6F1 | Protein names: Lmo1736 protein | Gene ontology: General function prediction only | Operon: Operon_304 | Hypothetical protein - RAST Product: Acetyltransferase |

**CAC99248**
Gene: lmo1170 | Loci: lmo1170 | p-value: 0.0052380970183 | Log2FC: 0.64 | FC: 1.6 | Entry: Q8P7U9 | Protein names: Lmo1170 protein | Gene ontology: Secondary metabolism, transport and catabolism | Operon: Operon_185 | Hypothetical protein - RAST Product: similar to Salmonella enterica Phad protein |

**CAC98683**

**CAD0083**
Gene: lmo310 | Loci: lmo310 | p-value: 0.01857356784700 | Log2FC: 0.64 | FC: 1.6 | Entry: Q8P9G3 | Protein names: Imidazole glycerol phosphate synthase subunit HisH (EC 2.4.2.1) | Gene ontology: transferase activity, transferring pentosyl groups | Operon: Operon_093 | Hypothetical protein - RAST Product: similar to amidotransferases with HisF IGPS catalysis the conversion of phosphoribulosyl-formimino-5-aminoimidazole-4-carboxamide ribonucleotide phosphate and glutamine to imidazole-glycerol phosphate and S-aminosuccinimide-4-carboxamide ribonucleotide, and glutamate in histidine biosynthesis the HisH subunit provides the glutamine amidotransferase activity that produces... |
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<td>metal ion binding phosphoribosyl-AMP cyclohydrolase activity [GO:0046872]; Amino acid transport and metabolism</td>
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<td>phosphoribosyl-AMP cyclohydrolase similar to phosphoribosyl-AMP cyclohydrolase [HuaA protein] FR-AMP cyclohydrolase functions in histidine biosynthesis from HMP converts L-(S)-phosphoribosyl-AMP to L-(S)-phosphoribosyl-5-(S)-phosphoribosylaminomethylphosphomonomethylphosphonic acid; dioxo-4-carboxymethyl during the histidine biosynthesis pathway binds zinc and magnesium forms homodimers</td>
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<td>Imidazole glycerol phosphate synthase subunit HisF</td>
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<td>Cell motility</td>
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<td>catalyzes the formation of (5)2-(5)-aminol-1-(5-phospho-D-ribosyl)</td>
<td>with PurU and PurQ catalyzes the conversion of formylglycinamidine ribonucleotide, ATP, and glutamine to formylglycinamidine ribonucleotide, ACP, and glutamate in the fourth step of the purine biosynthesis pathway</td>
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<td>catalyzes the formation of (5)2-(5)-aminol-1-(5-phospho-D-ribosyl)</td>
<td>with PurU and PurQ catalyzes the conversion of formylglycinamidine ribonucleotide, ATP, and glutamine to formylglycinamidine ribonucleotide, ACP, and glutamate in the fourth step of the purine biosynthesis pathway</td>
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**Downregulated genes in ∆rli47 vs wild-type**
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<td>Nucleotide transport and metabolism</td>
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<td>phosphoribosylformylglycinamidase synthase II</td>
<td>catalyzes the formation of 2-(formamido)-N1-(5-phospho-D-ribosyl)guanamine from N2-formyl-N1-(5-phospho-D-ribosyl)glycinamide and L-glutamine in purine biosynthesis</td>
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<td>phosphoribosylformylglycinamidase synthase I</td>
<td>similar to phosphoribosylformylglycinamidase synthase II catalyzes the formation of 2-(formamido)-N1-(5-phospho-D-ribosyl)guanamine from N2-formyl-N1-(5-phospho-D-ribosyl)glycinamide and L-glutamine in purine biosynthesis</td>
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Downregulated genes in ∆rli47 vs wild-type
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Table S5. Overlap between Rli47 regulon in DM and AgrA, σ^B, CodY and PrfA regulons.

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Titre: Étude des interconnexions dans les réseaux de régulation de *Listeria monocytogenes* en réponse aux conditions de l'environnement

**Mots clés:** *Listeria monocytogenes*, Agr, $\sigma^B$, Rli47, Réseaux de régulation

**Résumé:** *Listeria monocytogenes* est une bactérie ubiquiste présente dans des habitats variés. Le système de communication Agr et le facteur alternatif $\sigma^B$ sont essentiels pour l'adaptation et la survie de ce pathogène. L’objectif de ce travail était de décrypter les interconnexions entre les régulateurs AgrA et $\sigma^B$ dans le réseau de régulation de *L. monocytogenes*. Les données ont mis en évidence le rôle important de ces systèmes de régulation dans l’écologie de ce pathogène. Les résultats confirment la contribution des systèmes Agr et $\sigma^B$ pendant la croissance sessile de *L. monocytogenes*. Le ncARN Rli47, dépendant de $\sigma^B$ est un répresseur de la biosynthèse de l’isoleucine qui pourrait limiter la croissance de *L. monocytogenes* lorsque les conditions sont défavorables. Une meilleure compréhension des réseaux de régulation permettra de développer des stratégies de prévention de la croissance, de la survie et de la dissémination de *L. monocytogenes*.

Title: Overlaps in the regulatory networks of *Listeria monocytogenes* in response to environmental cues

**Keywords:** *Listeria monocytogenes*, Agr, $\sigma^B$, Rli47, Transcriptional regulation

**Abstract:** *Listeria monocytogenes* is a highly adaptable organism widely distributed in the environment and responsible for listeriosis, one of the leading causes of foodborne fatalities in developed countries. To adapt to stressful environments, this pathogen has developed mechanisms to quickly control gene expression, such as the Agr and $\sigma^B$ systems and even small non-coding RNA molecules. This thesis aimed to decipher the connections between Agr and $\sigma^B$ systems in *L. monocytogenes*. Results highlighted the important role that both systems play in the ecology of this pathogen and in biofilm formation. Moreover, the small RNA Rli47 was shown to specifically repress isoleucine biosynthesis as a way to restrict growth under harsh conditions, enhancing survival of *L. monocytogenes*. Understanding how stress signals are sensed and how gene expression changes to achieve a quick response may be a critical step in helping to develop strategies to prevent *L. monocytogenes* growth, survival and dissemination.