Biofilm formation and physiological heterogeneity of Listeria monocytogenes

Yue-Jia Lee

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Biofilm formation and physiological heterogeneity of \textit{Listeria monocytogenes}

By

Yue-Jia Lee

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Veterinary Medical Science
in the Department of Basic Sciences

Mississippi State, Mississippi

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Yue-Jia Lee

2019
Biofilm formation and physiological heterogeneity of *Listeria monocytogenes*

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A contributing factor in recurrent *Listeria monocytogenes* (*L. monocytogenes*) food contamination is that this bacterium produces biofilms on surfaces to persist in food-processing environments. Quorum sensing (QS) is a cell-to-cell communication system utilized by bacteria within biofilms to collaborate and adapt to environmental stresses. However, the details of how the QS-dependent network contributes to biofilm development of *L. monocytogenes* have yet to be well understood. By comparing the transfer rates of planktonic and biofilm (sessile) *L. monocytogenes* from stainless steel blades to bologna slices, we found that sessile bacteria had reduced transferability onto a single slice but caused the increase in the number of contaminated slices. This suggests that physiological adaptions derived during biofilm development affect bacterial dissemination. Given the contribution of proteins and environmental temperatures to the extracellular polymeric substances (EPS) synthesis and biofilm integrity, we evaluated the exoproteomes of biofilms formed at 25 and 37°C using 2D-gel electrophoresis and LC-MS/MS. We found exoproteases Lmo0186, Cwh, and Spl exclusively in biofilms formed at 25°C and their greater expression in the gene level at 25°C. By using the zymography and crystal-violet-staining assay with a protease inhibitor, we observed a greater proteolytic activity at lower temperatures and showed that the attenuated proteolytic activity of proteases is positively
correlated with increased biofilm-forming ability at 25°C. Considering the transcriptional role of QS systems during biofilm development, we investigated how the accessory gene regulator (Agr)-based and metabolite S-Adenosylmethionine (SAM)-involved QS systems modulate nutrient availability and EPS synthesis. The results revealed that the SAM signal interacts with the Agr QS at the transcriptional level during biofilm development, whereas SAM and Agr QS regulate distinct EPS synthesis pathways. Additionally, this interaction is dependent on bacterial life modes (planktonic and sessile). Overall, we conclude that *L. monocytogenes* manipulates the synthesis of EPS with the coregulation of metabolism and QS for biofilm formation and the production of exoproteases for biofilm dispersion. These precise regulations on EPS enable *L. monocytogenes* to prolong its survival and promote its dissemination in environments.
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CHAPTER I
INTRODUCTION

In the first part of this chapter, the readers can expect a general introduction of the physiological features of L. monocytogenes and Listeriosis. In the second part, more details about the biofilm and currently proposed mechanisms underlying biofilm development are provided. The details should guide the readers to comprehend the following studies of L. monocytogenes biofilms in this dissertation.

1.1 Listeria monocytogenes

1.1.1 Biological and taxonomic implications on environmental distribution

Listeria is a genus of facultative, rod-shaped, nonsporulating, gram-positive bacteria and currently comprised of 17 recognized species. When comparing genomic and phenotypic data, six species (L. monocytogenes, L. seeligeri, L. ivanovii, L. welshimeri, L. innocua, and L. marthii) that share common phenotypic properties have been placed into a distinct group named as Listeria sensu strictu. The shared phenotypic characteristics in Listeria sensu strictu include (1) replication at refrigerator temperatures (4 to 10°C), (2) flagellar motility at less than 30°C, (3) positive catalase reaction as observed by the generation of bubbles in hydrogen peroxide, (4) inability to reduce nitrate to nitrite, and (5) positive reaction in the Voges-Proskauer test, indicating the ability to produce acetoin from glucose fermentation through the butanediol pathway (Orsi & Wiedmann, 2016). L. monocytogenes and L. ivanovii are the two listerial species considered pathogenic because of their ability to invade host cells, replicate in the cytosol after escaping from
phagosomes, and spread to neighbor cells by polymerizing host actin. The most commonly isolated species from infected human cases is *L. monocytogenes*, which makes this pathogen being considered as a significant public health threat.

Based on serological reactions of somatic (O-factor) and flagellar (H-factor) antigens with specific antisera, *L. monocytogenes* strains are grouped into at least 13 serotypes. Since human listeriosis cases are mainly caused by three serotypes, 1/2a, 1/2b, and 4b, the value of serotyping isolates from suspected foods and clinical samples for epidemiological purposes is relatively low (Orsi, Bakker, & Wiedmann, 2011). To effectively trace and identify the source of a pathogen during a listeriosis outbreak, a variety of genotypic and phenotypic approaches have been developed and even been used for the identification of persisting clones in food processing environments (Nyarko & Donnelly, 2015). Results from a multilocus enzyme electrophoresis (MLEE) typing study helped to establish the first classification of two distinct phylogenetic divisions for *L. monocytogenes* isolates (Piffaretti et al., 1989). With further investigations and the improvement of sequencing techniques (Chen, Yi et al., 2016; Davis, S. et al., 2015; Salcedo, Arreaza, Alcala, de la Fuente, & Vázquez, 2003), four phylogenetic lineages are currently assigned for *L. monocytogenes* strains (Figure 1.1). Lineage I contains serotypes 1/2b, 3b, 3c, and 4b and is overrepresented among human isolates, while lineage II consists of serotypes 1/2a, 1/2c, and 3a, which are commonly isolated from food, food processing plants, and environments. Two additional lineages (III and IV) are composed of the isolates mostly obtained from ruminants, although strains in lineage IV are rarely announced in most studies.

Pieces of phenotypic data suggest that lineage II strains may generally be more adapted to a saprotrophic and environmental life mode because they have higher recombination rates compared to the strains in lineage I (den Bakker, Didelot, Fortes, Nightingale, & Wiedmann, 2008;
Orsi, Sun, & Wiedmann, 2008; Tsai, Y. H., Orsi, Nightingale, & Wiedmann, 2006). While some of lineage II isolates are virulence-attenuated due to premature stop codon mutations in virulence genes such as inlA and prfA (Olier et al., 2003; Roche et al., 2005; Velge et al., 2007), the structures of these virulence factors, such as truncated the InlA (Franciosa, Maugliani, Scalfaro, Floridi, & Aureli, 2009) and non-fully-transited form of PrfA (Lemon, Freitag, & Kolter, 2010; Miner, Port, Bouwer, Chang, & Freitag, 2008) that are specifically present when L. monocytogenes lives in environments are required for biofilm formation. These lines of evidence suggest that these virulence factors and similar proteins may be distinctly regulated in the expression, production or conformation by L. monocytogenes during the transition between different life modes. In Chapter III, the proteins that are related to biofilm development and produced differently based on L. monocytogenes life modes will be discussed.
Figure 1.1  Schematic summary of *L. monocytogenes* lineages and distribution.

*L. monocytogenes* isolates have been assigned to four lineages based on single nucleotide polymorphism (SNP) analysis of 3 chromosomal regions that include genes responsible for virulence, the stress response, and housekeeping functions. The prevalence of different ecological niches has also been linked to these lineages.
1.1.2 Growth characteristics and stress adaptation

As an environmental pathogen, *L. monocytogenes* can replicate and survive in both the environments and mammalian hosts (Xayarath & Freitag, 2012). *L. monocytogenes* is a robust bacterium with a relatively low demand for nutrients; however, it still requires external sources such as biotin, riboflavin, amino acids (the most required are cystine, valine, isoleucine, and leucine), and carbohydrates (glucose is the preferred source) for optimal growth (Premaratne, Lin, & Johnson, 1991; Tsai, H. N. & Hodgson, 2003). To some extent, this feature is reflected by the fact that *Listeria* spp. are frequently isolated from sites such as soil, vegetation, and food processing plants which provide a nutritional environment.

Other conditions such as the temperature between 30 and 37°C, the pH ranging from 6.0 to 7.0 and the water activity of 0.99 with NaCl are also preferred by *L. monocytogenes* for the optimal growth (Gray, M. L. & Killinger, 1966; O'Driscoll, Gahan, & Hill, 1996; Schwartzman, Belessi, Butler, Skandamis, & Jordan, 2011). However, it has been considerably reported that *L. monocytogenes* can survive in harsh environmental conditions, including a wide range of temperatures from – 0.1 to 45°C and pH values from 4.0 to 9.5, as well as the presence of high osmolality up to 10% (w/v) NaCl (Liu, D., Lawrence, Ainsworth, & Austin, 2005; McClure, Roberts, & Oguru, 1989; Phan-Thanh & Montagne, 1998; Walker, Archer, & Banks, 1990). This pathogen also persists for years with attachment and biofilm formation onto abiotic surfaces (Ferreira, Wiedmann, Teixeira, & Stasiewicz, 2014). These features explain the ubiquitous distribution of *L. monocytogenes* in a diverse range of environments, which results in a great hurdle for controlling this foodborne pathogen in the food industry.

For *L. monocytogenes*, it is possible that the same stresses take place on various occasions along the food supply chain to consumers. For example, the pathogen may encounter acidic and
osmotic stress first in the food matrix because of fermentation or food preservation and subsequently in the gastrointestinal tract of the host. In this respect, it is an important concept that resistance to different stresses is interconnected and supported by overlap mechanisms. Specifically, a number of resistance systems for environmental stresses are integrally mediated by the sigma subunit of RNA polymerase σB and are involved in many aspects of host-pathogen interactions (Chaturongakul et al., 2011; Mujahid, Orsi, Vangay, Boor, & Wiedmann, 2013; O'Byrne & Karatzas, 2008). Therefore, for *L. monocytogenes* and other gram-positive bacteria, σB is recognized as a central regulator for stress adaptation and an important mediator contributing to the switch between environmental and intracellular life mode (Gahan & Hill, 2014; Lauderdale, Boles, Cheung, & Horswill, 2009; van Schaik & Abee, 2005). Other examples of central regulators for the precise regulation of interconnected stress responses are CodY and AgrA which play an essential role in linking *L. monocytogenes* virulence to metabolism (Bennett et al., 2007) and biofilm formation (Garmyn, Augagneur, Gal, Vivant, & Piveteau, 2012), respectively. More details in the role of these central regulators that link metabolism to biofilm formation will be discussed in Chapter IV.

While gene regulation and bioreactions in the interior of the cell are indispensable to the stress responses, superficial contact is the most crucial element that provides the first line of defense for the sensing of and interaction with the cell exteriors. The ability to bind different compounds and to adhere to abiotic or biotic surfaces supports the survival of *L. monocytogenes* during its life cycle from free-living to host-associated status. Strongly depending on the composition and functionality of the cell envelope, this instinct is advantageous for biofilm formation and persistence in the environment (Carpentier & Cerf, 2011), for the colonization and internalization of host cells (Travier et al., 2013) and for the avoidance of immune responses
(Aubry et al., 2011; Witte et al., 2013). *L. monocytogenes* cell envelope consists of two functional layers: a cytoplasmic membrane and a thick cell wall surrounding the former (Rajagopal & Walker, 2017). The cell wall of *L. monocytogenes* is mainly composed of peptidoglycan (PG), teichoic acids (TAs), lipids and proteins that are enzymatic regulators for cell wall homeostasis as well as components of secretion and transport systems (Carvalho, Sousa, & Cabanes, 2014).

However, the cell envelope is also a vulnerable structure that is targeted by numerous antibiotics and host-secreted murolytic enzymes. Bacteria have developed mechanisms for peptidoglycan modification to interfere with these attacks (Davis, K. M. & Weiser, 2011; Vollmer, 2008). In terms of β-lactams targeting bacterial cell-wall synthesis, isolates of *L. monocytogenes* are naturally susceptible to penicillin and vancomycin but resistant to cephalosporins and fosfomycin (Hof, 2004), suggesting the lack of or low binding affinity with specific penicillin-binding proteins (PBPs) in the listerial cytoplasmic membrane (Guinane, Cotter, Ross, & Hill, 2006; Vicente, Pérez-Dá, Baquero, Angel de Pedro, & Berenguer, 1990; Zawadzka-Skomial et al., 2006). In addition to β-lactams, *L. monocytogenes* has different tolerance levels to antibiotics which are grouped by their inhibition on the synthesis of nucleic acid. Therefore, a currently recommended treatment for patients with invasive listeriosis is intravenous administration of broad-spectrum antibiotics such as penicillin or carbapenems accompanied with an aminoglycoside (Swaminathan & Gerner-Smidt, 2007; Thonnings et al., 2016).

1.1.3 **Listeriosis: from risk assessment to strategy development**

Listeriosis is an invasive infection resulted from the ingestion of food contaminated with *L. monocytogenes*. Although a case of invasive listeriosis is defined as isolation of *L. monocytogenes* (Centers for Disease Control and Prevention (CDC), 2013), rare cases of human infections with *L. ivanovii* (a pathogen of ruminants), *L. seeligeri* and *L. grayi* had been described
(Guillet et al., 2010; Rocourt, Hof, Schrettenbrunner, Malinverni, & Bille, 1986; Salimnia, Patel, Leхват, Fairfax, & Chandrasekar, 2010). The elderly, pregnant women, newborns, and immunocompromised individuals are particularly susceptible to listeriosis leading to bacteremia, meningitis, fetal loss, and death. According to a surveillance report in 2011, listeriosis is the third leading cause of death (19%) among major foodborne illnesses, compared with 0.5% for either *Salmonella* spp. or *E. coli* O157:H7 (Scallan et al., 2011). Despite recent efforts at prevention, listeriosis continues to be a threat for public health in the United States due to the ability of *L. monocytogenes* to survive in harsh environments such as low temperature, high osmolality, and low pH and to persist for years (Ferreira et al., 2014).

Food contamination is of particular concern for ready-to-eat foods because there is no heat treatment or other antimicrobial processes between production and consumption (Srey, Jahid, & Ha, 2013). Results from risk assessment studies indicate that deli meats pose the highest risk (>5 cases per billion servings) for listeriosis, accounting for approximately 1,600 illnesses per year (Swaminathan & Gerner-Smidt, 2007; U.S. Department of Agriculture Food Safety and Inspection Service, 2003). The group composed of high-fat dairy products, soft unripened cheeses, pasteurized milk, and fresh soft cheeses is the group second likely to cause listeriosis. Moreover, approximately 83% of cases related to deli meats occurred at deli counters during slicing and packaging (U.S. Department of Agriculture Food Safety and Inspection Service, 2010). Therefore, further knowledge about the causes for the high risk of cross-contamination between slicers and deli meats is necessary to control the outbreak of listeriosis and to develop effective hygiene practices. Chapter II aims to provide details in the transferability of *L. monocytogenes* from the slicer to beef bologna.
The infectious dose in invasive listeriosis is difficult to determine because of variability in host susceptibility and food matrix effects. It has been estimated that the approximate infective dose of *L. monocytogenes* is 7 to 9 log colony forming units (CFU) in healthy hosts, and only 5 to 6 log CFU in susceptible individuals (Farber, Ross, & Harwig, 1996). Despite a low probability of developing listeriosis when consuming below 100 CFU *L. monocytogenes*, based on the known characteristics of *L. monocytogenes* and its life-threatening infection, the Food and Drug Administration (FDA) maintains a policy of “zero-tolerance” for *L. monocytogenes* in ready-to-eat foods since the 1980s. This means that there is no acceptance of the detection of any *L. monocytogenes* in either of the two 25-gram tested samples of food (U.S. Department of Agriculture Food Safety and Inspection Service, 2003).

Listeria are ubiquitous in the environment and use many potential avenues to enter the food facility (Todd & Notermans, 2011). Furthermore, *L. monocytogenes* has a tendency to adhere to and generate biofilms on a variety of surfaces including metals, rubbers, and polymers, which are approved materials commonly used in food-processing equipment (Beresford, Andrew, & Shama, 2001; Silva, Teixeira, Oliveira, & Azeredo, 2008). A mature biofilm can be established by *L. monocytogenes* after 24-hour inoculation at 25°C on a stainless steel surface (Rieu et al., 2008) (The details in biofilm formation will be described in section 1.2). Therefore, it is important to assess potential routes of *L. monocytogenes* dissemination and transmission to know the locations that need to be intensively monitored and cleaned. It is also worth to investigate how different processes and environmental conditions affect bacterial growth at various stages of food processing.

Since the shelf lives of most food products implicated in listeriosis are extended, time and temperature during processing, transport, and storage primarily govern the risk of listeriosis.
associated with RTE foods (Buchanan, Gorris, Hayman, Jackson, & Whiting, 2017). Based on the results of risk assessment and existing dose-response models, rules and criteria have been established by governing bodies such as USDA-FSIS and Health Canada for controlling *L. monocytogenes* in postlethality exposed RTE foods (after the step of lethality treatment such as cooking and fermentation) (Farber, Kozak, & Duquette, 2011; U.S. Department of Agriculture Food Safety and Inspection Serice, 2014). For instance, the effective administration of antimicrobial agents and processes should allow no more than 2-logs of growth of the pathogen over the shelf life of the product. To achieve the criteria set by the “zero tolerance” policy, (1) use of natural preserving additives and sanitizers (alkaline-, acid-, alcohol- and QAC-based products), (2) cold storage, and (3) separation of cutting and cooling rooms in processing environment and retail are considered as fundamental strategies for preventing cross-contamination with *L. monocytogenes* (David, Steenson, & Davidson, 2013; Kurpas, Wieczorek, & Osek, 2018; Muhterem-Uyar et al., 2015). In Chapter II, the effect of the cold storage on the growth of transferred *L. monocytogenes* from the slicer to beef bologna will be discussed.

Therefore, tracing the frequently isolated *L. monocytogenes* strains and understanding *L. monocytogenes* stress responses are expected to provide insight into the evolution and mechanisms underlying the persistence of this foodborne pathogen and open an avenue of opportunities to advance food safety and listeriosis control.
1.2 Biofilm life mode

1.2.1 Biofilm’s structure and stepwise development

Biofilm-forming ability and the acquisition of resistance mechanisms in *L. monocytogenes* (Holch et al., 2013; Lunden, Autio, Markkula, Hellstrom, & Korkeala, 2003) at unhygienic harborage sites (Carpentier & Cerf, 2011) are main factors contributing to *L. monocytogenes* persistence in food processing environments (Martínez-Suárez, Ortiz, & López-Alonso, 2016). Biofilms are structured communities of microbial cells embedded in self-produced extracellular polymeric substances (EPS) in association with a surface (Donlan & Costerton, 2002). EPS can account for over 90% of the dry matrix of biofilm and are recognized as the hallmark of biofilm formation (Flemming & Wingender, 2010).

For certain microorganisms such as *Staphylococcus* and *Pseudomonas*, EPS are primarily composed of exopolysaccharides (Sadovskaya, Vinogradov, Flahaut, Kogan, & Jabbouri, 2005; Wingender, Strathmann, Rode, Leis, & Flemming, 2001). However, extracellular proteins are the most abundant exopolymers within *L. monocytogenes* EPS (Combrouse et al., 2013; Frølund, Palmgren, Keiding, & Nielsen, 1996). Given the essential role of superficial proteins in the adhesion and biofilm formation of *L. monocytogenes*, Longhi et al. (2008) and Nguyen and Burrows (2014) evaluated the effect of protease treatments and demonstrated that the treatments abolish biofilm development and reduce established biofilms to undetectable levels in biofilm-forming *L. monocytogenes*. Additionally, the inactivation of secretion system and deletion of genes responsible for exoproteins, which lead to the absence of extracellular proteins, dramatically impact biofilm architecture (Renier, Chagnot, et al., 2013) and biofilm-forming ability (Franciosa et al., 2009; Lourenço et al., 2013). All these data suggest that extracellular proteins within EPS strongly contribute to *L. monocytogenes* biofilm development and structures.
The four steps sequentially repeating in the process of biofilm development are reversible attachment, irreversible attachment, maturation and detachment (Figure 1.2). Reversible attachment as the first contact with surfaces is dependent on the physicochemical and electrostatic interactions between planktonic bacteria and the substrate of the surface. Both environmental and bacterial factors are the determinants for successful contact and entry into the stage of irreversible attachment. For instance, adhesins that are located on the bacterial cell surface or cellular appendages such as flagellar and fimbriae facilitate the irreversible attachment (Lemon, Higgins, & Kolter, 2007; Proft & Baker, 2009).

Following the initial attachment, bacterial cells proliferate and grow into aggregated and sessile colonies. During biofilm maturation, bacteria start producing extracellular matrix known as EPS and communicate by synthesizing and detecting quorum sensing (QS) molecules (Landini, Antoniani, Burgess, & Nijland, 2010; Tuson & Weibel, 2013). At this stage, bacteria living in the biofilm undergo cell differentiation and become phenotypically different from their planktonic counterparts regarding to growth rate, gene expression, and protein production (Guilhen et al., 2016; Mata, da Silva, Wilson, Lowe, & Bowman, 2015; Sauer, Camper, Ehrlich, Costerton, & Davies, 2002). For example, the bacteria can alter the expression of genes for higher tolerance to antimicrobial treatments (Chavant, Gaillard-Martinie, & Hébraud, 2004; Folsom et al., 2010; Kostaki, Chorianopoulos, Braxou, Nychas, & Giaouris, 2012; Mah & O'Toole, 2001). Recent evidence further suggests that regulatory networks consisting of small noncoding RNAs are required for drug tolerance and antimicrobial resistance in biofilms (Borgmann, Schäkermann, Bandow, & Narberhaus, 2018; Chambers & Sauer, 2013; Nishino, Yamasaki, Hayashi-Nishino, & Yamaguchi, 2011).
From mature biofilms, a part of bacterial cells in sessile communities (surface-attached) switch to planktonic (free-floating) growth where cells rapidly multiply and disperse to colonize new surfaces, leading to the dissemination of bacteria in environments. Therefore, dispersion is not only the last stage of biofilm development but also the beginning of a new one. Bacteria can leave original biofilms by “active dispersion”, which depends on internal changes caused by environmental stresses and by “passive dispersion”, which depends on external physical factors such as shearing force and abrasion (Kaplan, J. B., 2010; McDougald, Rice, Barraud, Steinberg, & Kjelleberg, 2012). The effectors that support active dispersion include (1) the synthesis of enzymes and surfactants for degrading biofilm matrix (Baty, Eastburn, Techkarnjanaruk, Goodman, & Geesey, 2000; Boles, Thoendel, & Singh, 2005), (2) the induction of motility with reduced c-di-GMP signal (Barraud et al., 2009) and (3) the presence of bacteriophages and genes with homology to cell lysis (Rossmann et al., 2015).

Coexistence of sessile and planktonic cells on the surface has been demonstrated in different bacterial species (Houry et al., 2012; López & Kolter, 2010; Marchal et al., 2011). Furthermore, distinct characteristics of these bacterial cells result in local physiological and genetic heterogeneities within biofilms. In terms of the risk to human public health, physiological heterogeneity developed during biofilm development confers bacteria advanced resistance to antimicrobial agents and high motility (Chua et al., 2013; Liu, J., Ling, Zhang, & Wu, 2013) and thus accounts for the failure of disinfection procedures and antimicrobial treatments.
Biofilm development is a dynamic process that includes four discernable steps: reversible and irreversible attachment, maturation, and dispersion. Planktonic, sessile, and dispersed cells are in gray, green, and yellow, respectively.
1.2.2 Peptidoglycan synthesis and regulation: the foundation of EPS synthesis

The biosynthesis and remodeling of the cell wall are the foundation of EPS synthesis for bacterial physiological events including biofilm development and infection (Davis, K. M. & Weiser, 2011; Mercier et al., 2002; Popowska, 2004; Typas, Banzhaf, Gross, & Vollmer, 2011). In the cell wall, peptidoglycan (PG) is the major constituent and acts as a biological scaffold for the surface anchoring of proteins and glycopolymers with relevant physiological roles. PG is a linear or branched polymer of repeating disaccharide units containing N-acetylmuramic acid (MurNAc)–(β-1,4)-N-acetylglucosamine (GlcNAc) and is cross-linked by peptide bridges. The gram-positive PG sacculus is generally interspersed with a family of secondary glycopolymers called teichoic acids (TAs) that can either be tethered to the plasma membrane (lipoteichoic acids, LTA) or be covalently linked to the sugar backbone of the PG matrix (wall TAs, WTA)(Neuhaus & Baddiley, 2003).

In principle, the synthesis of PG begins with an intracellular process utilizing nucleoside diphosphate sugars which are later conjugated with a chain of amino acids. This process continues with the transportation of PG precursors which are exported from the cell. Most of the bacteria conserve the canonical pathway of PG biosynthesis that takes place in three overall stages (Figure 1.3A). First, in the cytoplasm, the soluble, activated nucleotide precursors UDP-MurNAc are converted from UDP-GlcNAc by two enzymes MurA and MurB. GlcNAc and are cross-linked with pentapeptide by a series of four enzymes, namely, the Mur ligases (MurC, D, E, and F). (Barreteau et al., 2008). Second, at the inner membrane, the soluble precursor UDP-MurNAc-pentapeptide are conjugated with the undecaprenyl phosphate (carrier lipid) and UDP-GlcNAc to form the monosaccharide- and disaccharide-anchored lipids (lipid I and II) by the proteins MraY and MurG. Then they are flipped across the membrane by members of the SEDS (shape,
elongation, division, sporulation) family of proteins, namely RodA and FtsW (Bouhss, Trunkfield, Bugg, & Mengin-Lecreulx, 2008; Mohammadi et al., 2011; Müller, Klöckner, & Schneider, 2017). Third, the glycan of lipid II is polymerized, and undecaprenyl pyrophosphate is recycled for the next cycle of PG synthesis. The resulting glycan strands are polymerized and inserted into the sacculus to generate mature PG.

PG maturation requires PG synthases which are categorized as (1) glycosyltransferases (GTases) for polymerizing the glycan chains and (2) transpeptidases (TPases, also known as PBPs) for crosslinking the peptides (Korsak, Markiewicz, Gutkind, & Ayala, 2010; Vollmer & Bertsche, 2008). On the other hand, PG hydrolases are necessary for the cleavage of both glycoside and amide bonds, which can elongate but not thicken the sacculus with the insertion of new glycan strands (Popowska, 2004; Vollmer, Joris, Charlier, & Foster, 2008). Given that the enzymatic steps in the biosynthetic pathway are well-conserved across both gram-negative and gram-positive bacteria, the enzymes in PG synthesis are targets for many clinically used antibiotics.

A number of PG synthases and hydrolases (at least ten PBPs, six NAGases, four NAMases, four amidases, and three endopeptidases) have been predicted in the genome of L. monocytogenes (Bierne & Cossart, 2007; Renier, Micheau, Talon, Hébraud, & Desvaux, 2012). Only have a few been experimentally validated, but these experimental results highlight the role of PG synthesis in L. monocytogenes biofilm formation and pathogenicity (Korsak et al., 2010; Machata, Hain, Rohde, & Chakraborty, 2005; Pinto, São-José, Santos, & Chambel, 2013; Popowska, 2004; Rismondo et al., 2015). PG hydrolases are grouped based on the cleavage site of PG. NAGases and NAMases break the glycosidic bonds on the GlcNAc and MurNAc residues, respectively. Amidases are responsible for the separation of the peptide from the sugar strand by cleaving the
amide bond between MurNAc and L-alanine, whereas endo- and carboxypeptidases hydrolyze the amide bonds within and between peptide bridges (Vollmer et al., 2008).

At present, four general mechanisms are demonstrated for EPS synthesis in bacteria: (1) the Wzx/Wzy-dependent pathway (in alignment with the enzymatic steps of PG synthesis), (2) the synthase-dependent pathway, (3) the ATP-binding cassette (ABC) transporter-dependent pathway, and (4) the extracellular synthesis with a single sucrase protein (Schmid, Sieber, & Rehm, 2015).

Unique to L. monocytogenes, the Pss complex encoded by the pssA-E operon in combination with a separately located pssZ gene also plays a role in peptidoglycan biosynthesis for EPS production (Figure 1.3B). Assembling in the Pss Complex, PssD moves the growing polysaccharide chain onto the cell surface, PssZ acts as a PG hydrolase to cleave glycosidic bonds of PG, and PssB may modify PG through deacetylation (Köseoğlu et al., 2015). The formed polysaccharide chains by the Pss complex may be incorporated into the peptidoglycan structure to create cell wall glycopolymers by unknown mechanisms or similar pathways found in other gram-positive bacteria (Brown, Santa Maria, & Walker, 2013; Weidenmaier & Peschel, 2008). The level of c-di-GMP modulated by diguanylate cyclases (DGCs) and phosphodiesterases (PDEs) has also been confirmed as an important signal to activate the Pss complex through the binding of c-di-GMP to the subunit PssE (Chen, L. H. et al., 2014). This specific set of biosynthetic proteins for EPS synthesis in L. monocytogenes appears to share similar characteristics with synthase-dependent systems; however, the details remain unclear.
Figure 1.3  Schematic representation of the biosynthetic machinery for EPS synthesis in *L. monocytogenes*.

(A) A canonical synthesis of peptidoglycan (PG) which is the major component of EPS takes place in three overall stages. It starts in the cytoplasm with the conversion of UDP-N-acetylglucosamine (UDP-GlcNAc) into the soluble precursor UDP-N-acetylmuramic acid-pentapeptide (UDP-MurNAc-pentapeptide), which is sequentially catalyzed by the Mur enzymes. The second step is the assembly and translocation of the lipid II precursor. MraY transfers UDP-MurNAc-pentapeptide to the undecaprenyl phosphate (lipid carrier) to form lipid I and then MurG adds a UDP-GlcNAc on lipid I to produce lipid II (undecaprenyl phosphate-GlcNAc-MurNAc-pentapeptide). Sequentially, lipid II is translocated across the membrane through FtsW/RodA, proteins of SDES family. In the third step, the glycan of lipid II is incorporated into the PG sacculus by PG synthases and hydrolases including penicillin-binding proteins (PBPs) for transglycosylation (TG) and transpeptidation (TP) reactions. 

(B) *L. monocytogenes* exclusively possesses the machinery of EPS synthesis, which relies on the Pss complex with the subunit PssE. The activation of Pss complex requires the binding of the subunit PssE with c-di-GMP molecule synthesized by diguanylate cyclase DgcA. The activated Pss complex translocates the MurNAc precursors and galactoses in the cytoplasm to form polysaccharide chains outside the cells. The peptidoglycan and polysaccharide chains are both parts of the cell wall glycopolymers. Figure modified according to Köseoğlu et al. (2015) and Müller et al. (2017).
1.2.3 Regulatory machinery underlying biofilm development

The formation of biofilm structures varies among bacterial species and is dependent on environmental conditions. This indicates that the bacterial sessile life mode is governed by multiple pathways and the same pathway with its cooperators can determine different fates of biofilm development (Tolker-Nielsen, 2015). Cell-to-cell communication mechanisms (such as quorum sensing) and the intracellular level of the second messenger cyclic di-GMP (c-di-GMP) are known as determinant regulators in bacterial biofilm development.

1.2.3.1 QS circuits and their physiological roles in multiple facets

Despite differences in components and cooperant molecular mechanisms, all known QS circuits basically include the synthesis, secretion, and detection of small signal molecules. Once these extracellular signal molecules are sensed, intracellular signal transduction induces gene transcription to modify the cellular physiology for subsequent responses to the changing environmental conditions. QS signaling molecules are produced by the members of the community so that the production and detection of signaling molecules were found to be highly related to the density of population (Kaplan, H. B. & Greenberg, 1985). However, the action of QS system does not reflect the cell density in some bacteria such as L. monocytogenes and Pseudomonas aeruginosa, suggesting that the primary function of QS circuits in these bacteria is not to monitor population density (Garmyn et al., 2011; McKnight, Iglewski, & Pesci, 2000; Wen, J., Karthikeyan, Hawkins, Anantheswaran, & Knabel, 2013).

QS circuits are categorized according to the type of the signal molecule and the machinery used to integrate the signal. Currently, the most-studied QS circuits include three classes of signal molecules, which are (1) cyclic peptides (autoinducing peptide, AIP) for gram-positive bacteria, (2) acyl-homoserine lactones (AHL) for gram-negative bacteria, and (3) furanone-derived
autoinducers (AI) for gram-positive and gram-negative bacteria. In terms of the permeability, AHL can freely diffuse in and out of a cell (Kaplan, H. B. & Greenberg, 1985; McKnight et al., 2000), while AIP requires a secretion or transport system for their exportation from a cell (Thoendel & Horswill, 2010) and are typically detected by a membrane-bound two-component system (TCS). TCSs generally work in the way where a histidine kinase receptor (first component) binds to AIP and auto-phosphorylates its cognate cytoplasmic response regulator (second component) to activate the transcription of downstream genes (Havarstein, Coomaraswamy, & Morrison, 1995; Ji, Beavis, & Novick, 1995).

Two known QS circuits have been described in L. monocytogenes (Figure 1.3). One is encoded by the accessory gene regulator (agr) locus – lmo0048-0051 (agrBDCA). This Agr-based quorum sensing system (Agr QS) is driven by the signal molecule AIP paired with a classical TCS for signal transduction. As coming from the precursor peptide AgrD, AIP is processed and secreted by the membrane-binding peptidase AgrB. The TCS in Agr QS, which consists of the receptor AgrC and regulator AgrA, responds to the presence of extracellular AIP to regulate L. monocytogenes attachment to surfaces (Rieu, Weidmann, Garmyn, Piveteau, & Guzzo, 2007) and pathogenicity (Autret, Raynaud, Dubail, Berche, & Charbit, 2003; Riedel et al., 2009). Although the virulence genes controlled by Agr QS and the autoregulated transcription of the agr locus have been widely studied (Garmyn et al., 2012; Pinheiro et al., 2018; Riedel et al., 2009), our understanding of the transcriptional regulation of PG synthesis and EPS construction by Agr QS is still limited.

Staphylococcus aureus has a similar Agr-based QS as L. monocytogenes does but it uses this circuit to inhibit the biofilm formation through decreasing adhesions and increasing proteases with the regulation of a dual-functional RNA (RNAIII) (Bronesky et al., 2016; Lauderdale et al.,
that has not been found in \textit{L. monocytogenes}. This indicates that the same QS can generate different outputs depending on coexisting regulators. Interestingly, in spite of going through different QS circuits, behavior such as RNA-inhibiting biofilm formation in \textit{Staphylococcus aureus} is analogous to the strategy used by \textit{Vibrio cholerae} which expresses quorum-regulatory sRNAs (Qrr1-4) to target two QS regulators (Lenz et al., 2004; Rutherford, van Kessel, Shao, & Bassler, 2011). Overall, these studies revealed the species-specificity and complexity of QS circuits.

The other QS system found in \textit{L. monocytogenes} involves the signal molecule AI-2. In this system, two enzymes encoded by \textit{lmo1494} (\textit{pfs}) and \textit{lmo1288} (\textit{luxS}) catalyze the conversion of S-Adenosylhomocysteine (SAH) into S-Ribosylhomocysteine (SRH) and sequentially into homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD). Homocysteine is continually converted to methionine and then to S-Adenosylmethionine (SAM) for completion of the activated methyl cycle (AMC), while DPD spontaneously cyclizes into various furanone derivatives called AI-2.

Previous studies reported increased biofilm formation in \textit{luxS}-deficient mutants of \textit{L. monocytogenes}. However, complementation of the mutant strains with exogenous AI-2 failed to restore wild-type levels of biofilm formation (Challan Belval et al., 2006; Sela, Frank, Belausov, & Pinto, 2006) and AI-2 receptors have not been discovered, which make the QS role of AI-2 in \textit{L. monocytogenes} biofilm formation controversial. Specifically, the enzyme LuxS functions as both a synthase for the precursor of AI-2 and an integral enzyme in the AMC. The dual function of the LuxS implies an interconnection between bacterial metabolism and LuxS-dependent biofilm formation. Therefore, the existence of central regulators for balancing nutrient availability with LuxS-based or other QR circuits have been proposed, and certain regulators were characterized in
some bacteria. Taking *Bacillus cereus* as an example, the CodY regulator controls multicellular behavior in different facets, including the induction of biofilm development under nutrient limitation and enhancing expression of motility and virulence factors when nutrients are abundant (Lindback et al., 2012). CodY also has a similar role in *Staphylococcus aureus* where it delays QS response until rich nutrients are available (Pohl et al., 2009) and in *L. monocytogenes* where CodY has a positive effect on the induction of the *agr* operon (Bennett et al., 2007; Garmyn et al., 2012). The example in which CodY mediates the gene transcription for Agr QS implies that a response by Agr QS to nutrient availability may exist in *L. monocytogenes*, although the detailed mechanism remains unclear.
Figure 1.4  Schematic diagram of two QS systems in *Listeria monocytogenes*.  

The **left** is the generation of the QS signal molecule (autoinducer AI-2) based on LuxS, which combines with the activated methyl cycle (AMC) for methionine recycle and *S*-Adenosylmethionine (SAM) synthesis (SAH: *S*-Adenosylhomocysteine, SRH: *S*-Ribosylhomocysteine, Pfs: *S*-Adenosylhomocysteine nucleosidase, LuxS: *S*-Ribosylhomocysteinase, MetE: methyltransferase, MetK: *S*-Adenosylmethionine synthetase). The **right** shows the accessory gene regulator (*agr*) locus-encoding QS, where AgrD is processed by AgrB to form the QS signal molecule (autoinducing peptide AIP) and AIPs activate the two-component system AgrCA with the binding to AgrC. (AgrD: AIP precursor, AgrB: AIP-processing endopeptidase, AgrC: kinase receptor, and AgrA: response regulator)
1.2.3.2 Noncoding RNA and small molecule are interconnectors for QS circuits

Transcriptional regulators, noncoding RNAs, and small molecules have been found to act cooperatively in *L. monocytogenes*. The transcription factor PrfA is well known for its temperature-dependent role in *L. monocytogenes* virulence and intracellular infection (Johansson et al., 2002; Loh et al., 2009). Recently, PrfA was found to positively impact biofilm development in *L. monocytogenes* which lives outside mammalian hosts by transcriptional and posttranslational regulations (Lemon et al., 2010). The stress response factor σB can recognize the P2_{prfA} promoter region and induce the transcription of *prfA* (Rauch, Luo, Muller-Altrock, & Goebel, 2005; Schwab, Bowen, Nadon, Wiedmann, & Boor, 2005). It was speculated that σB-induced *prfA* expression partly contributes to σB-dependent biofilm formation at temperatures lower than mammalian body temperatures (Lemon et al., 2010). The precursor of the QS molecule AgrD also has a positive correlation to the expression of *prfA* (Garmyn et al., 2012). However, the translation of the *prfA* transcript can be prevented by binding with the riboswitch SreA (if presented), as the conformation of transcribed RNA is relatively stable at low temperatures compared to the body temperature. On the other hand, the presence of SreA induces the expression of *agrD* (Loh et al., 2009; Riedel et al., 2009). At the posttranslational level, the protein conformation of PrfA, which is not fully transited to the intracellular activity form, plays a role in promoting *L. monocytogenes* biofilm-forming ability with yet-known mechanisms (Lemon et al., 2010). Based on the known roles of PrfA, σB and Agr QS in both biofilm development and host invasion, one can speculate that these regulators are the determinants, possibly intermediated by SreA, for *L. monocytogenes* switching between environmental and intracellular life mode based on surrounding temperatures and nutrient availability.
The secondary messenger c-di-GMP is an example of small molecules that acts as a central regulator contributing to the switch between the sessile and planktonic life mode in various bacterial species (Almblad et al., 2015; Ryan et al., 2006; Zhao, Koestler, Waters, & Hammer, 2013). In general, high c-di-GMP concentration in the cell lead to biofilm formation, while low c-di-GMP levels caused by the activation of QS circuits are the sign for motility and biofilm dispersion (Petrova & Sauer, 2016; Purcell & Tamayo, 2016). With respect to *L. monocytogenes*, Chen, L. H. et al. (2014) revealed that overexpression of c-di-GMP induces the synthesis of EPS, which leads to cell aggregation, enhanced resistance to disinfectants and slightly reduced biofilm formation. C-di-GMP-inhibited biofilm formation was later supported by Piercey, Hingston, and Truelstrup Hansen (2016) who found enhanced biofilm formation in the mutant with the deletion of *lmo0531* (*pssE*) encoding a c-di-GMP receptor in the Pss complex (Chen, L. H. et al., 2014). It appears that c-di-GMP and QS signaling in *L. monocytogenes* function in the different or even opposite way of their known roles in other bacteria.

Apparently, the results mentioned above suggest that a part of determinants for virulence are indispensable for biofilm development. These factors are usually global regulators manipulating the transition between environmental and intracellular life modes to provide an exquisite control mechanism for responses to harsh conditions, such as environmental temperature shift. Although numerous pathways administered by global regulators for biofilm development and other cellular adaptation have been revealed, the regulatory networks with these regulators for *L. monocytogenes* biofilm development in the environment remain to be elucidated. Chapter IV concludes the currently proposed regulatory networks for *L. monocytogenes* biofilm development based on published data from other groups and the results in this dissertation.
1.3 Specific aims

The overall objective of this dissertation was to understand the mechanisms and regulatory networks underlying the survival strategy of *L. monocytogenes* to form biofilms in the environment. To fulfill this objective, we hypothesized that the cross-effect of responses developed during biofilm formation renders sessile *L. monocytogenes* different physiological properties through QS circuits and multiple signaling pathways. The following specific aims were proposed to address the overall objective:

(1) **Understand the growth, transferability, and morphological change in *L. monocytogenes* during biofilm development.**

Stainless steel is the most commonly used material for food-processing equipment and facilities; thus, blades or chips made of stainless steel will be used as the surfaces for biofilm formation in this study. Physiological changes, including morphology, transferability, and growth during the refrigerated storage of *L. monocytogenes* in different life modes, will be investigated using SEM, mimicking bologna slicing in retail outlets, and using general biological assays to give insight into specific attributes of *L. monocytogenes* dissemination and persistence.

(2) **Compare the exoproteomes of *L. monocytogenes* biofilms formed at 25°C and 37°C to identify the exoproteins involved in biofilm development.**

According to previous findings, extracellular proteins within EPS undoubtedly play a crucial role in bacterial cell wall turnover and biofilm development. Two-dimensional gel electrophoresis and LC-MS/MS will be used to identify unique proteins present in exoproteomes of biofilms formed at either 25° or 37° C. Functional assays will be further performed to characterize the role of identified exoproteins in biofilm development.
(3) Investigate the role of the activated methyl cycle and its connection to the Agr quorum sensing system in *L. monocytogenes* biofilm development.

The Agr QS combining multiple global regulators has been proposed to form a regulatory network for *L. monocytogenes* biofilm development and adaptation to environmental stresses. To map this complex network in detail, the biofilm assay and qPCR will be performed to investigate the coeffects of interconnection between S-Adenosylmethionine activated methyl cycle and Agr QS on biofilm volumes and gene expressions for EPS synthesis in *L. monocytogenes*.
CHAPTER II
CHARACTERIZATION OF PHYSIOLOGICAL PROPERTIES OF BIOFILM-FORMING *LISTERIA MONOCYTOGENES*

2.1 Introduction

Listeriosis is an invasive infection usually resulting from ingestion of food contaminated with gram-positive *Listeria monocytogenes*. According to surveillance in 2011, listeriosis is the third leading cause of death (19%) among major foodborne illnesses (Scallan et al., 2011). Despite recent efforts at prevention, listeriosis continues to be a public health threat in the United States owing to the ability of *L. monocytogenes* to survive in harsh environments such as low temperature, high osmolality, and low pH, and to persist for years (Ferreira et al., 2014).

Ready-to-eat (RTE) foods are convenient, but they are usually considered a relatively high-risk route to cross-contamination and foodborne illness because the products are consumed directly without cooking or undergoing any bactericidal process (Srey et al., 2013). According to a 2003 risk assessment study, deli meats pose the greatest risk for listeriosis, accounting for ~1,600 illnesses per year (U.S. Department of Agriculture Food Safety and Inspection Serice, 2003), with a high percentage of cases related to deli meats sliced at deli counters (U.S. Department of Agriculture Food Safety and Inspection Serice, 2010). Based on these studies, further knowledge about the
reasons for the high risk of cross-contamination between slicers and deli meats is necessary to control the outbreak of listeriosis and to develop effective hygiene practices.

A contributing factor for *L. monocytogenes* to survive and persist in a processing environment is its ability to form biofilms (Bridier et al., 2015; Ferreira et al., 2014; Lewis, 2005; Norwood & Gilmour, 2001), that is, microbial communities that confer resistance to antimicrobial agents and protection from environmental stresses (López, Vlamakis, & Kolter, 2010). Numerous studies have been conducted on the relationship between biofilm formation and attachment on abiotic surfaces by using various strains of *L. monocytogenes* with weak or strong biofilm-producing ability (Hansen & Vogel, 2011; Jordan et al., 2008; Keskinen, Todd, & Ryser, 2008; Rodríguez, Autio, & McLandsborough, 2007). However, the outcomes were too inconsistent and inconclusive to precisely understand the effects of biofilms on *L. monocytogenes* cross-contamination in food processing plants (Ferreira et al., 2014).

Given that the biofilm is a unique mode of bacteria distinct from its planktonic counterpart, and that the cross-contamination of RTE meats frequently occurs during slicing, we hypothesized that biofilms that form on the surface of slicers might play a significant role in the high risk for cross-contamination of RTE meats. Thus, we evaluated the growth of biofilms on stainless steel surfaces as well as the effect of biofilms on the transfer rate of *L. monocytogenes* from inoculated stainless steel blades to beef bologna. With a better understanding of the physiology of the *L. monocytogenes* biofilms and its effect on cross-contamination, we expect to gain further insight into biofilm-related contamination and its mechanisms.
2.2 Materials and methods

Bacterial strain and culture conditions

*L. monocytogenes* EGD-e is serovar 1/2a; it accounts for > 50% of *L. monocytogenes* isolates recovered from foods and the environment (Aarnisalo et al., 2003; Gilbreth et al., 2005a). The EGD-e strain was grown in brain heart infusion plates (Difco, BD, Sparks, MD) overnight from a frozen stock and then transferred to brain heart infusion broth and incubated for 16 h at 30°C.

Preparation of stainless steel coupons and blades

Stainless steel coupons (0.75 × 0.75 × 0.075 in [1.9 by 1.9 by 1.9 cm], type 304, #4 finish; Stainless Supply Inc., Monroe, NC) and stainless steel blades (EdgeCraft, Avondale, PA) were washed and then autoclaved at 121°C for 15 min. Sterilized coupons were used for biofilm assay with crystal violet staining and for investigation of biofilm formation with scanning electron microscopy (SEM).

Biofilm formation

*L. monocytogenes* EGD-e was grown in brain heart infusion broth for 16 h and diluted to $10^5$ or $10^7$ CFU/ml. For the biofilm assay, 5 ml of diluted bacterial culture were added to one well of a six-well sterile polystyrene tissue culture microtiter plate (CELLTREAT Scientific Products, Pepperell, MA) containing one stainless steel coupon per well. The plates were incubated at 25°C for 1 h and for 1, 3, 5, 7, or 14 days. After incubation, the production and structure of the biofilm were evaluated using the crystal violet staining assay and SEM, respectively. For the transferability test, 400 ml of diluted bacterial culture were added to a glass tray containing a sterile blade and incubated at 25°C for 5 min or 3 days for attachment or biofilm formation on the blades, respectively.
To prevent evaporation, all the plates and trays were wrapped with plastic wrap before incubation.

**Scanning electron microscopy**

After the inoculation described above, the stainless steel coupons were removed from the well and washed with 5 ml of sterile double-distilled water three times to remove any loosely attached cells. Then, the coupons were immersed in a fixing solution (1/2 Karnovsky's: 2.5% glutaraldehyde and 2% formaldehyde in 0.1M sodium cacodylate buffer) for at least 24 h at room temperature. After rinsing and postfixation for 1 h with 2% osmium tetroxide, specimens were washed four times with double-distilled water, dehydrated using a graded ethanol series (35, 50, 70, 70, 95, 95, 100, 100, 100 and 100% for 15 min each), and then chemically dried with hexamethyldisilazane in ethanol (25, 50, 75, 100 and 100% for 15 min each). After air-drying overnight, specimens were mounted on aluminum stubs with double-sided carbon tape, coated with 15-nm Platinum and observed with a JSM-6500F scanning electron microscope (JEOL, Tokyo, Japan). All chemicals used for SEM were purchased from Electron Microscopy Sciences (Hatfield, PA). The protocol was conducted as described by Fischer, Hansen, Nair, Hoyt, and Dorward (2012), with minor modifications as needed.

To quantitate morphological changes, we measured the length (longest dimension parallel to the coupon surface) and width (shortest dimension parallel to the coupon surface) of horizontally attached *L. monocytogenes* EGD-e on the coupons at the different time points mentioned above. Then, we used the ratios of cellular length to width as the indication of morphological changes.
Biofilm assay

After 1 h and 1, 3, 5, 7, and 14 days of incubation, the biofilms formed on the stainless steel coupons were detected using the crystal violet staining method, with modifications as previously described (Borucki, Peppin, White, Loge, & Call, 2003). In brief, the coupons were washed three times with sterile phosphate-buffered saline (PBS; Sigma, St. Louis, MO) to remove the planktonic cells and then dried for 30 min. Biofilms on the stainless steel coupons were stained by adding 3 ml of a 0.1% crystal violet solution (Remel, Lenexa, KS) in 20% ethanol to each well of six-well plastic plates for 30 min at room temperature. The unbound dye in the well was removed by rinsing three times with 5 ml of sterile double-distilled water followed by drying for 30 min. Crystal violet was solubilized in 2 ml of 95% ethanol for 30 min at room temperature with 100 rpm agitation. The contents of each well (200 µl) were then transferred to a sterile polystyrene microtiter plate, and the optical density at 595 nm of each well was measured using a Synergy HT microplate reader (BioTek, Winooski, VT).

Transfer of L. monocytogenes EGD-e from stainless steel blades to beef bologna

A commercial electric food slicer (model 609 C; EdgeCraft) equipped with nonserrated stainless steel blades was used for slicing. The blades were inoculated with $10^5$ or $10^7$ CFU/ml L. monocytogenes EGD-e per blade in a total of 400 ml. The two durations of inoculation for each initial concentration were 5 min and 3 days, which were designed to simulate attachment of planktonic L. monocytogenes EGD-e (without the biofilm) and growth of biofilm with sessile L. monocytogenes EGD-e on the blade, respectively. After inoculation, beef bologna purchased from a local retailer was mechanically sliced to obtain 15 slices that were each ~ 3 mm in thickness and weighed ~
30 g. Beef bologna sliced by sterile blades was used as a negative control to ensure sterile technique for each experiment. Each slice was placed in a sterile Whirl-Pak sampling bag (Nasco, Fort Atkinson, WI) and weighed. This packaging was followed by the addition of buffered peptone water (Sigma) at a ratio of 1:1 (wt/vol). The bag containing a slice of beef bologna was homogenized in a Stomacher 400 (Seward, Davie, FL) for 1 min. The homogenate was diluted appropriately in PBS, and 100-µl aliquots of diluent were plated on Oxford agar (Sigma). After 2 days of incubation at 30°C, colonies on the plates were counted and the number of *L. monocytogenes* EGD-e per slice was recorded. Transfer rate was defined as the percentage of cells transferred from the donor blade to the recipient (total 15 slices) and can be written as follows: Transfer rate = CFU recipient/CFU donor × 100 (Chen, Yuhuan, Jackson, Chea, & Schaffner, 2001). The bags with slices and buffered peptone water were further stored aerobically at 4°C for 7 days, and bacterial enumeration was performed at days 4 and 7. Each experiment was repeated at least three times.

**Statistical analysis**

The experiments were a factorial 2 × 2 design (i.e., the presence or absence of biofilm and two initial levels of inoculation, 10^5 or 10^7 CFU/ml). Bacterial enumeration data were converted to log CFU and are presented as means ± standard deviations. Significant differences were assessed by two-way analysis of variance using SigmaPlot (Systat Software, San Jose, CA). Multiple comparisons were performed using Tukey’s test. For all statistical tests, a *P* value of < 0.05 was considered significantly different.
2.3 Results

2.3.1 Biofilm-producing capacity of *L. monocytogenes* EGD-e grown on stainless steel coupons.

Biofilm formation of *L. monocytogenes* EGD-e on the surface of stainless steel coupons was investigated using SEM and crystal violet staining. Based on SEM images, *L. monocytogenes* EGD-e attached rapidly to the surface of stainless steel coupons in 1h (Figure 2.1A and 2.1G) and formed a thin monolayer of biofilm (Figure 2.1B and 2.1H) containing extracellular polymeric substances–like substances (Figure 2.1b and 2.1h) in 1 day. After 3 or 5 days, depending on the initial concentration of inoculation, a mature biofilm with a honeycomb-like structure was observed (Figure 2.1D and 2.1I). The $10^7$-CFU/ml initial concentration of inoculation lead to earlier formation of the mature biofilm (in 3 days) than the $10^5$-CFU/ml concentration. The biofilm formation of *L. monocytogenes* EGD-e was also evaluated using the crystal violet staining assay that detects the amount of biofilm matrix (Figure 2.2). These assay data were compatible with the results from SEM (Figure 2.1). During 14 days of incubation at 25°C, the maximum biofilm volume was observed on the third and fifth day of incubation for the $10^7$- and $10^5$-CFU/ml inoculation conditions, respectively. Thereafter, the production of biofilm decreased gradually in both groups with different initial concentrations of inoculation. Furthermore, the SEM images revealed that *L. monocytogenes* EGD-e carried out binary fission vigorously before a mature biofilm was formed (Figure 2.1b, 2.1c, and 2.1h). Then, they changed their cellular morphology from rod to sphere after 5 days ($10^7$ CFU/ml) or 7 days ($10^5$ CFU/ml) of incubation (Figure 2.1e, 2.1f, 2.1j, 2.1k, and 2.1l), as we observed that the ratios of cellular length to width were $3.5 \pm 0.9$ and $4.5 \pm 1.2$ on the
first day and then significantly decreased to $1.9 \pm 0.8$ and $1.7 \pm 0.5$ on the seventh and fifth days for initial concentrations of $10^5$ and $10^7$ CFU/ml inoculation, respectively.
Figure 2.1  Scanning electron microscopy (SEM) of biofilm formation of *Listeria monocytogenes*.

*L. monocytogenes* at an initial concentration of $10^5$ (A through F, a through f) or $10^7$ (G through L, g through l) CFU/ml in brain heart infusion buffer were grown at 25°C on stainless steel coupons for 1 h (A, a, G, g), 1 day (B, b, H, h), 3 days (C, c, I, i), 5 days (D, d, J, j), 7 days (E, e, K, k), or 14 days (F, f, L, l). A surface without inoculation of *L. monocytogenes* after 14 days is shown as a negative control (M). SEM images were taken at $1.0 \times 10^4$ (A through L) and $1.0 \times 10^5$ (a through l, M) magnification. Cellular binary fission, extracellular polymeric substances, and sphere-shaped *L. monocytogenes* are indicated by an arrow, arrowhead, and asterisk, respectively.
Figure 2.2  Biofilm-producing capacity of *Listeria monocytogenes* grown on stainless steel coupon at 25°C.

Five milliliters of brain heart infusion buffer with $10^5$ or $10^7$ CFU/ml *L. monocytogenes* was added into each well of a six-well plate containing a stainless steel coupon (2 × 2 cm) to form the biofilm at 25°C. Biofilm production was evaluated using the crystal violet assay. The data represent means ± standard deviations from three independent experiments for each time point. (n≥3)

2.3.2 Transfer of *L. monocytogenes* EGD-e from inoculated stainless steel blades to uninoculated beef bologna.

Immersing stainless steel blades in brain heart infusion broth that was inoculated with *L. monocytogenes* EGD-e for 5 min or 3 days was implemented to simulate contamination of food processing equipment without or with a biofilm, respectively. The concentrations of *L. monocytogenes* EGD-e detected from the blades after 5-min inoculation were 3.1 and 5.2 log CFU for initial concentrations of $10^5$ and $10^7$ CFU/ml inoculation, respectively. After slicing with the contaminated blade without the biofilm (5-min inoculation), the quantities of transferred *L. monocytogenes* EGD-e on the first slice of beef bologna were $2.63 \pm 0.24$ and $4.57 \pm 0.42$ log CFU from the blades.
inoculated with $10^5$ and $10^7$ CFU/ml *L. monocytogenes* EGD-e, respectively (Figure 2.3A). Regardless of the initial concentration of inoculation on the blades, the amount of transferred *L. monocytogenes* EGD-e detected on the third slice was 100-fold less than that on the first slice. As more slices were cut off, the amount of transferred *L. monocytogenes* EGD-e on the slice decreased gradually after the third slice. Specifically, none was recovered from the 15th slice contaminated by the blade with $10^5$ CFU/ml of initial inoculation. Log difference values were used to compare the inoculation concentrations, calculated as the log CFU on the slice produced by the blade contaminated with $10^5$ CFU/ml subtracted from that on the slice produced by the blade contaminated with $10^7$ CFU/ml. Comparing the amount of transferred *L. monocytogenes* EGD-e on the corresponding slices, the difference values ranged from 2 to 3 log CFU per slice throughout all sequential slices. These values corresponded to the 2-log CFU/ml difference between $10^5$ and $10^7$ CFU/ml of initial inoculation, and they were not significantly different.

Three days after inoculation, the concentrations of *L. monocytogenes* EGD-e detected from the blades before slicing were similar between the two groups with different initial concentrations of inoculation (~5.6 log CFU per blade). After slicing, the amounts of initially transferred *L. monocytogenes* EGD-e on the first slice were also similar to each other: $2.67 \pm 0.73$ and $3.22 \pm 0.14$ log CFU per slice from blades inoculated with $10^5$ and $10^7$ CFU/ml of *L. monocytogenes* EGD-e, respectively (Figure 3B). However, it was interesting to observe that the overall transfer rates among 15 slices were significantly different between the two bacterial inoculation times (Figure 4). Planktonic *L. monocytogenes* EGD-e, which attach to the blades during 5-min
inoculation, have a greater ability of transfer from the stainless steel blades to bologna slices, compared with the sessile *L. monocytogenes* EGD-e, which exist within the biofilm formed after 3 days of contamination (*P* = 0.006). This result is independent of the initial level of inoculation (*P* = 0.133).

![Figure 2.3](image)

**Figure 2.3** Transfer of attached or biofilm-forming *Listeria monocytogenes* from inoculated slicer blades to uninoculated beef bologna.

Stainless-steel blades were inoculated with 10⁵ or 10⁷ CFU/ml *L. monocytogenes* for 5 min (A) to attach to blades or 3 days (B) to form the biofilm on the blades. Transfer of *L. monocytogenes* was quantified by plating homogenates on modified Oxford agar. Data are shown as means ± standard deviations (log CFU per slice) for three to four replicates. The asterisk indicates that every amount of transferred *L. monocytogenes* on each of the 15 slices contaminated by blades inoculated with 10⁷ CFU/ml was significantly higher than that on the counterpart slice contaminated by blades inoculated with 10⁵ CFU/ml.
Transfer rates of attached and biofilm-forming *Listeria monocytogenes* on the first 15 slices of beef bologna.

Transfer rate = CFU recipient/CFU donor × 100. Values were presented as means ± standard deviations of three to four independent experiments. The asterisks indicate statistically significant differences (*P* < 0.05) in comparisons between groups.

### 2.3.3 Growth rates of *L. monocytogenes* EGD-e on bologna slices during 4°C storage.

Across all four experimental groups, the amount of transferred *L. monocytogenes* EGD-e enumerated from each of the 15 slices increased after 7 days of storage at 4°C, with no significant differences (*P* > 0.05) among the growth rates (defined by the ratio of end-day CFU per slice to first-day CFU per slice) of transferred bacteria (Figure 2.5). Notably, although nothing was initially detected on the 15th slice cut by the blade inoculated with 10⁵ CFU/ml for 5 min, *L. monocytogenes* EGD-e was detected after 4 days of storage at 4°C (Figure 2.5A). The same-level and horizontal data lines shown in...
Figure 2.5 indicate that the growth rate was independent of the initial concentration of inoculation (10^5 versus 10^7 CFU/ml) and the amounts of transferred *L. monocytogenes* EGD-e on the slices. Also, the two gray lines that represent the growth rates in the earlier and later period of storage almost overlap, indicating that the growth rate of transferred *L. monocytogenes* EGD-e remained the same over the time of storage.

The slices contaminated by blade inoculated with 10^5 (A, C) or 10^7 (B, D) CFU/ml *L. monocytogenes* for 5 min (A, B) or 3 days (C, D) were stored at 4°C for a total of 7 days. Growth of transferred *L. monocytogenes* was evaluated by plating homogenates on modified Oxford agar. Data represent the ratio of end-day CFU to first-day CFU in the first 4 days, first 7 days, and last 3 days during storage. Data are means ± standard deviations for three to four replicates.
2.4 Discussion

Several food safety-related studies on the transferability of *L. monocytogenes* during slicing indicate that the transfer rate of *L. monocytogenes* can be influenced by various environmental and intrinsic factors. These factors include characteristics of the contact surface (Midelet & Carpentier, 2002; Vorst, Todd, & Rysert, 2006); ambient conditions such as temperature (Aarnisalo, Sheen, Raaska, & Tamplin, 2007) and relative humidity (Hansen & Vogel, 2011; Rodríguez et al., 2007); and bacterial strain and physiological state, which include the presence or absence of biofilms (Hansen & Vogel, 2011; Midelet, Kobilinsky, & Carpentier, 2006). Despite considerable progress in controlling Listeria outbreaks, the complexity of *L. monocytogenes* biofilm makes the development of efficient strategies against biofilm-related contamination in the food industry a giant challenge. This partly explains why listeriosis incidence has not decreased as expected since 2001 (Crim et al., 2014; Silk et al., 2012). In this study, our goal was to quantify *L. monocytogenes* transfer with or without biofilm during mechanical slicing of bologna with a stainless steel blade. Knowledge gained from our present investigation on the properties of listerial biofilms on stainless steel and their relationship with bacterial transferability could help assess key targets and develop novel approaches to control listerial cross-contamination in the food processing environment.

Previous studies showed that depending on the strains, interfacial hydrophobicity, and experimental setup used, *L. monocytogenes* biofilm takes on various forms, including a monolayer of adherent cells, flat unstructured multilayers, a honeycomb-like morphotype, and a knitted-chain network (Borucki et al., 2003; Guilbaud, Piveteau, Desvaux, Brisse, & Briandet, 2014; Marsh, Luo, & Wang, 2003; Rieu et al., 2008).
this study, we did observe a honeycomb-like structure similar to the description by Marsh et al. (2003). In addition, we noted that mature biofilms formed 3 or 5 days after inoculation, developed into microcolonies, produced extracellular polymers, and formed fimbria-like structures that protruded from the cells (Figure 2.1). These observations are all consistent with previous findings (de Oliveira, Brugnera, Alves, & Piccoli, 2010; Renier, Hébraud, & Desvaux, 2011).

To our knowledge, our study is the first to report the occurrence of spherical \textit{L. monocytogenes} EGD-e in late-stage biofilm on stainless steel surfaces, although this phenomenon was previously observed on a glass surface by Trémoulet, Duché, Namane, Martinie, and Labadie (2002). The spherical shape of \textit{L. monocytogenes} in late-stage biofilms on stainless steel surfaces has not been observed in other studies (Chavant, Martinie, Meylheuc, Bellon-Fontaine, & Hebraud, 2002; Zameer, Gopal, Krohne, & Kreft, 2010). This discrepancy could be due to low temperatures resulting in greater cell length (Chavant et al., 2002), apart from a morphological change from rod to sphere, in \textit{L. monocytogenes}. Although the biological implication of increased \textit{L. monocytogenes} length under low temperature is unclear, based on previous studies, this morphological change is potentially a result of physiological adaptation to specific environmental stresses including starvation (Miladi, Ammar, Ben Slama, Sakly, & Bakhrouf, 2013; Persat et al., 2015; Wen, Jia, Anantheswaran, & Knabel, 2009). Understanding the role and mechanism of altered morphology in biofilm development is an important next step to further comprehend the unique properties of bacterial biofilm.

To evaluate the impact of biofilm on listerial attachment and transfer rate, we inoculated \textit{L. monocytogenes} EGD-e for 5 min or 3 days on stainless steel blades to
establish models of attached or biofilm-featured *L. monocytogenes* EGD-e, respectively. We observed a pattern of progressive reduction in the quantity of transferred *L. monocytogenes* EGD-e in 15 consecutive slices of bologna; especially, we noted a sharp decrease in the first five slices (Figure 2.3). These results are in agreement with previous studies in which the initial level of inoculation on the slicer blade and working environment were cited to be the two main contributing factors for listerial transferability (Chen, D., Zhao, & Doyle, 2014; Sheen & Hwang, 2008). We also noted that the overall transfer rate among 15 slices was significantly inhibited by the presence of biofilm (Figure 2.4). We suppose that the decreasing amount of transferred *L. monocytogenes* EGD-e on the first slice is the main contributor to our observed inhibitory effect of the biofilm on the overall transfer rate, although the mean of decreasing amount had high standard deviation and its value was susceptible to slicing conditions. This result may also be explained by how differences in the capillary effect make biofilm-featured cells less available for transfer, as shown by Hansen and Vogel (2011). Most importantly, we confirm that biofilm-featured *L. monocytogenes* EGD-e with decreasing transfer rate can release cells slowly for an extended period, leading to persistent contamination of RTE foods, as alluded to in a review by Srey et al. (2013).

Several studies indicate that sessile cells within biofilms have intrinsically distinct characteristics from their planktonic counterparts because these two forms exhibit extraordinarily different patterns of genetic and proteomic profiles (Belessi, Gounadaki, Schwartzman, Jordan, & Skandamis, 2011; Hefford et al., 2005; Mata et al., 2015). In addition, biofilm-featured sessile cells remain in a slow, but active growth state for eliciting stress responses at stringent conditions.
Based on the altered transfer rates noted in our study, we hypothesized that *L. monocytogenes* EGD-e transferred from biofilm-formed blades grow slower than their planktonic counterparts during low-temperature storage. To test this hypothesis, we enumerated transferred *L. monocytogenes* EGD-e on bologna slices that had been stored at 4°C for 7 days. Contrary to our expectations, both the intrinsic properties of sessile cells in biofilms and the initial levels of transferred bacteria on the slices did not influence the growth rate of transferred bacteria during the 7-day storage at 4°C (Figure 2.5). This shows that once the food has been contaminated by food processing equipment, transferred *L. monocytogenes* EGD-e were able to survive steadily on food at low temperature regardless of cellular biofilm-featured properties and initial concentration on the slices, even if *L. monocytogenes* EGD-e was undetectable before storage. A possible explanation for the observed similarity in growth rates of transferred bacteria is that the adaptive mechanisms of *L. monocytogenes* to low temperature may lead to decreased or compromised growth regulation.

In summary, our study elucidated the potential role of a morphological modification that may be involved in biofilm development for strengthening *L. monocytogenes* resistance to environmental stresses. In addition, frequently occurring listeriosis outbreaks may be explained by a scenario whereby a decreasing transfer rate related to biofilm formation gives rise to increasing risks of (i) false-negative diagnosis because the bacterial load is under the detection limit and of (ii) cross-contamination for an extended period because more bacteria remain on the slicer by each cut. Our results provide useful information for the prevention of cross-contamination between slicers and foods. However, future studies on the role of morphological modification in biofilm
development and on how to improve the sensitivity of detection of pathogens in foods are needed to ensure the safety of food products for consumers.
CHAPTER III
THE ROLE OF EXTRACELLULAR PROTEASES IN LISTERIA MONOCYTGENES CELL
WALL TURNOVER AND BIOFILM FORMATION

3.1 Introduction

Listeriosis is a serious disease caused by ingestion of food contaminated with *L. monocytogenes*. This disease leads to 15-20% of annual food-related deaths worldwide and is especially dangerous for the elderly, fetus, newborns, pregnant women, and immunocompromised patients (European Food Safety Authority; European Centre for Disease Prevention and Control, 2016; Scallan et al., 2011). *L. monocytogenes* is capable of adhering to multiple abiotic surfaces (Beresford et al., 2001; Silva et al., 2008) and forming biofilms, which increases the probability of contamination between food processing equipment and foods (Lee & Wang, 2017). During biofilm development, some *L. monocytogenes* cells within biofilms are released into the surrounding environment through the process of dispersion. The releasing of bacteria with advanced resistance to sanitizers is a great concern for the food processing industries (Chavant et al., 2004; van der Veen & Abee, 2010).

Biofilms are surface-associated microbial communities that confer resistance to antimicrobials and protection against environmental stresses. Biofilm formation is a dynamic process whereby microbes first attach to a surface, produce extracellular polymeric substances (EPS) that immobilize the biofilm structure, and finally disperse from the surface for the next cycle of biofilm formation at a new location (Hall-Stoodley & Stoodley, 2002). Bacteria undergo
profound physiological changes during their transition from a planktonic life mode to a sessile, biofilm-associated life mode and then to a dispersed, free-floating life mode (O'Toole, Kaplan, & Kolter, 2000). Sessile cells and dispersed cells have been reported to be highly tolerant to antimicrobial agents and highly virulent to immune cells compared with their planktonic cells (Chua et al., 2014; Pan, Breidt, & Kathariou, 2006; Stewart et al., 2015; Uppuluri et al., 2010). Therefore, characteristics acquired during biofilm development make the removal of biofilms difficult and aggravate the cross-contamination from food processing equipment to foods (Manios & Skandamis, 2014).

In addition to the intrinsic adaptations of bacteria, EPS composed of exopolysaccharides, exoproteins, extracellular DNA, and lipids contribute substantial protection to biofilm communities (Flemming & Wingender, 2010). Exoproteins are the most abundant exopolymers within listerial EPS (Combrouse et al., 2013; Frølund et al., 1996). Additionally, previous studies reported that protease treatments abolish biofilm development or reduce established biofilms to undetectable levels (Longhi et al., 2008; Nguyen & Burrows, 2014). Those results suggest that exoproteins play a key role in L. monocytogenes biofilm formation and are thus potential targets for developing strategies to prevent and remove listerial biofilms.

Exoproteins within EPS vary in composition and quantity according to environmental conditions. Since L. monocytogenes forms biofilms mainly in environments where temperatures are different from those inside host cells, we hypothesized that the components of its biofilm exoproteomes would change with environmental temperatures. To test that hypothesis and investigate links between exoproteins and biofilm development, we compared the exoproteomes of L. monocytogenes biofilms formed at 25°C, a temperature that is encountered in food processing environments, with those formed at 37°C, the temperature inside host cells.
3.2 Materials and Methods

Bacterial strain and culture conditions

*L. monocytogenes* strain EGD-e, a serovar 1/2a strain, was used in this study, as serovar 1/2a accounts for > 50% of *L. monocytogenes* isolates recovered from foods and the environment (Aarnisalo et al., 2003; Gilbreth et al., 2005b). Frozen stock of this strain was kept at -80°C and subcultured in brain heart infusion broth (BHI; Difco, Sparks, MD) for 16 h at 37°C for following experiments.

Preparation of stainless-steel coupons

Stainless steel chips (2 × 2 × 0.2 cm, type 304, #4 finish; Stainless Supply, Inc., Monroe, NC) were used to mimic the materials used in food processing equipment. The chips were initially washed with absolute ethanol, rinsed with distilled water, air dried and then autoclaved at 121°C for 15 min.

Biofilm growth

Overnight cultures of *L. monocytogenes* strain EGD-e were diluted to 10⁷ CFU in Welshimer's broth (MWB; HiMedia Laboratories LLC, West Chester, PA). Five milliliters of bacterial diluent or blank MWB (control) were added to six-well sterile polystyrene microtiter plates (CELLTREAT, Pepperell, MA) that were preloaded with a single stainless steel chip per well. The plates were incubated at 25°C for 24 h and then transferred to either 20, 25 or 37°C for an additional 48 h incubation. The broth was replaced by fresh broth once every 24 h during the incubation.

For protease inhibitor assays, overnight cultures were diluted to 10⁷ CFU in MWB containing 0, 5, 50, 500, or 1000 µM of protease inhibitor 4-(2-Aminoethyl)-benzenesulfonylfluoride hydrochloride (AEBSF, Gold Biotechnology, Olivette, MO). Bacteria
treated with 500 ng/ml TPCK-treated trypsin (Thermo Fisher Scientific, Waltham, MA) in the presence or absence of 500 µM AEBSF served as positive controls. We added 250 µl bacterial diluents or blank MWB (negative control) to 96-well sterile polystyrene microtiter plates and incubated them at 25 or 37°C for 24 h.

**Crystal violet staining**

Crystal violet staining was applied to quantify the biofilm biomass in protease inhibitor assays as described by Lourenço, Rego, Brito, and Frank (2012) with minor modification. In brief, the biofilms formed in wells as described above were dried for 30 min after the liquid suspension was removed from the well. The wells were then stained with 100 µl 0.1% crystal violet solution with 20% ethanol for 30 min at room temperature. The unbound dye in the well was removed by rinsing the wells three times with 100 µl sterile double-distilled water. Crystal violet was then solubilized in 100 µl 95% ethanol for 30 min at room temperature with 100 rpm agitation. The optical density at 595 nm (OD$_{595}$) was measured using a Synergy HT microplate reader (BioTek, Winooski, VT).

**Separation of sessile and dispersed cells**

After 3 days of incubation, the two cultures of biofilms were separated into two fractions to collect distinct populations of sessile and dispersed cells. The suspensions from the biofilm cultures were removed and centrifuged at 2,800 g for 30 min. The pellets (comprising the dispersed cells) were washed with sterile phosphate-buffered saline (PBS) and centrifuged at 5,000 g for 10 min, while the supernatants were kept for further protein extraction. After the suspensions were removed from the biofilm cultures, the sessile cells were those remained on the stainless steel surface. We flushed the chips thoroughly with fresh MWB to remove the sessile cells and centrifuged them at 2,800 g for 10 min.
Protein extraction and quantification

Proteins were extracted from the suspensions of the biofilm cultures after the removal of bacterial cells using the phenol extraction method as described by Faurobert, Pelpoir, and Chaïb (2007). The collected supernatants were loaded onto 10 kDa-cut-off concentrators and centrifuged at 2,800 g at 4°C for 30 min. The concentrated supernatants were desalted with an equal-to-original volume of PBS. A final concentration of 200 µM phenylmethylsulfonyl fluoride (PMSF, Sigma, St. Louis, MO) was added to the protein concentrates to inhibit protease activities. The protein concentrates were mixed with extraction buffer (0.5 M Tris-HCl pH 8.8, 50 mM EDTA, 0.9 M sucrose, 0.1 M KCl, 2% β-mercaptoethanol, 1 mM PMSF) and Tris-phenol (pH 8.0) at a ratio of 1:4:1. After 10-min of shaking and 10 min of centrifugation at 7,500 g at 4°C, the phenol phase was collected and re-treated with extraction buffer at a ratio of 1:4. The recovered phenol phase was then treated with a precipitation solution (0.1 M ammonium acetate in methanol) at a ratio of 1:4 and incubated overnight at -20°C. The crude proteins were pelleted and washed three times with the cooled precipitation solution and another three times with 80% acetone. The resultant pellets were dried and stored at -80°C for further analysis. Protein concentrations were determined using the Bradford Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

2D-PAGE was performed as previously described by Renier, Chambon, et al. (2013). For isoelectric focusing (IEF), 75 µg protein extracts were mixed with IEF buffer [7M urea, 2M thiourea, 4% (w/v) CHAPS, 1% DTT, 0.2% ampholytes with pH 3-10] to make a total volume of
200 μL. The mixtures were loaded on immobilized pH gradient (IPG) strips with a pH 3-10 nonlinear gradient or a pH 4-7 linear gradient (Bio-Rad, Hercules, CA). The strips were subjected to rehydration and IEF for a total of 35,000 vh (12 h at 50 V, 15 min at 250 V, linear gradient to 8000 V over 2.5 h, and 8000 V until the end) at 23°C in a reswelling tray within a PROTEAN IEF cell (Bio-Rad). The strips were equilibrated in an equilibration solution (6M urea, 0.375M Tris-HCl pH 8.8, 2% SDS, 20% glycerol) containing 2% (w/v) dithiothreitol (DTT) for 15 min and in 2.5% (w/v) iodoacetamide (IAA) for another 15 min. The second-dimension electrophoresis (SDS-PAGE) was carried out with 10 % acrylamide gel in a mini-Protean system (Bio-Rad). The obtained 2D gels were stained overnight with 0.5% (w/v) Coomassie Blue G250 in 45% (v/v) methanol and 10% (v/v) acetic acid and scanned using a ProteomeWorks Spot cutter (Bio-Rad).

**2D-PAGE gel image and statistical analysis**

Images of six 2D-PAGE gels (three for the pH 3-10 nonlinear gradient and three for the pH 4-7 linear gradient) from three independent experiments were analyzed for each experimental temperature using PDQuest software, version 8.1 (Bio-Rad). Normalization using local regression method was applied to calibrate all the sample data with those of the reference gel (representing exoproteomes at 25°C). A Student's t-test was applied to determine significant changes in protein spot intensities ($P < 0.05$). Spots that were exclusively present in exoproteomes formed at either 25 or 37°C were selected for peptide identification.

**Peptide identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS)**

In-gel tryptic digestion was carried out using the In-Gel Tryptic Digestion Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s instruction with minor modification. The spots of interest were cut into 1 mm × 1 mm pieces and de-stained using 200
µL de-staining solutions (100 mM NH₄HCO₃, 50% methanol, 50% water and 25 mM NH₄HCO₃, 50% acetonitrile, 50% water) at 37°C and for 30 min in each de-staining solution. We dehydrated the gels with 100% acetonitrile for 30 sec and dry them for 5 min. In-gel proteins were reduced using 50 mM Tris[2-carboxyethyl]phosphine at 60°C for 10 min and then alkylated by 100 mM IAA in the dark at room temperature for 1 h. The gel pieces were dehydrated with acetonitrile and further treated with 10 µL activated trypsin solution (10 ng/µL) for 15 min and subsequently with an extra 25 µL digestion buffer (25 mM NH₄HCO₃ in DDW). The resultant samples were digested overnight at 30°C with agitation. The supernatants were cleaned up using Pierce C18 Spin Columns (Thermo Fisher Scientific) according to the manufacturer’s instructions. The resultant samples were dried and stored at −20°C until use.

The peptide samples were shipped to the Arizona Proteomics Consortium and analyzed using an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) equipped with an Advion nanomate ESI source (Advion, Ithaca, NY). Tandem mass spectra were searched against the protein database Listeria_UniprotKB_021118_Cont.fasta (98952 entries). Scaffold v 4.8.4 (Proteome Software Inc., Portland, OR) was used to validate the MS/MS-based protein and peptide identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.9% probability and contained at least five identified peptides.

**Bioinformatic analysis**

Protein identification data from Scaffold were imported into the knowledge-based LGER proteome database (http://leger2.gbf.de/cgi-bin/expLeger.pl) which provides the
functional annotation of proteins (Dieterich, Karst, Fischer, Wehland, & Jansch, 2006) and predicts the subcellular localization of proteins (Renier et al., 2012).

**RNA extraction and reverse transcription-quantitative PCR (RT-qPCR)**

To quantify the mRNA expression of target genes, total RNA was extracted from the bacterial pellets using acid phenol-chloroform extraction (Chomczynski & Sacchi, 2006) combined with the RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany). The cell pellets were resuspended in lysis buffer (15 mg/ml lysozyme and 200 µg/ml Proteinase K in TE buffer) and incubated at 37°C for 10 min. The resultant samples were transferred to lysing matrix B tubes (MP Biomedicals, Santa Ana, CA) and vortexed for 15 sec for four times using a disruptor (Scientific Industries, Bohemia, NY) with a 1-min pause on ice between vortexes. The mixture of phenol/chloroform/isoamyl alcohol (25:24:1) was added into the samples and centrifuged at 16,000 g at 4°C for 10 min. The aqueous phase was then treated with 100% chloroform and centrifuged at 16,000 g at 4°C for 10 min. Contaminating DNA was eliminated and total RNA was purified using RNeasy Plus Universal Mini Kit according to the manufacturer’s instructions. In addition, RNase-free DNase (Promega, Madison, WI) was applied to the samples at 37°C for 15 min after samples were loaded onto the columns. The purity and concentration of the RNA were determined using gel electrophoresis and a Nanodrop ND1000 UV-visible light spectrophotometer (Thermo Fisher Scientific). One microgram aliquot of RNA samples was reverse-transcribed to cDNA using the SuperScript VILO cDNA synthesis kit (Qiagen). cDNAs diluted by a factor of 5, 10, or 20 were used as the template in a 10-µl-volume qPCR reaction with the primers for cwh (F: GAGCCGTGGATGTTATCGTATTTAAC; R: GTAACGGACCAACTACATTTGATTGC), spl (F: AGGCTATAAGGTTTTCCTAGTTGTG; R: TAGTTCGGATACCTCTACACCAAG), lmo0186 (F:
AACACCAGTTTCTAAGGTATCC
TTC; R: GGATCAACCGCAATTACTTTTAGTTCC), and pbpA1 (F:
AGAGTACACGGGAGAAAATGCTCAATAC; R: TGGTTTCATAGTAGACCCCAACAGA).
qPCR was performed using the SYBR Green Master Kit (Applied Biosystems, Foster City, CA) and the 7500 Fast Real-Time PCR system (Applied Biosystems) under the following conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. 16S RNA was used as an internal control. The relative changes of mRNA expression were analyzed by the $2^{-\Delta\Delta Ct}$ method.

**Gelatin zymography**

Protease activity of the concentrated proteins from the supernatants of the biofilm cultures formed at 20, 25 or 37°C was analyzed by zymography using 0.1% gelatin (Sigma, St. Louis, MO) as the substrate under non-reducing conditions as previously described (Löwer et al., 2008). The supernatants of the biofilm cultures from each temperature were mixed with 5× zymogram sample buffer [125 mM Tris-base, 4% (w/v) SDS, 20% (v/v) glycerol, 0.01% (w/v) bromophenol blue] at a ratio of 4:1. We used 2.5 μg/ml trypsin as a positive control and 2 mg/ml bovine serum albumin (BSA) as a negative control. After SDS-PAGE, the gel was washed in renaturing buffer (2.5% Triton X-100, 0.5M Tris-HCl, 0.2M NaCl, 5mM CaCl$_2$) for 30 min twice at room temperature and transferred to developing buffer (0.5M Tris-HCl, 0.2M NaCl, 5mM CaCl$_2$, pH 7.5) for 10 min at 37°C with 100 rpm agitation. The gel was then incubated in fresh developing buffer at 37°C for 24 h. Lytic bands appeared as translucent zones in the opaque gel that was stained with 0.5% (w/v) Coomassie Blue G250 in 45% (w/v) methanol and 10% (w/v) acetic acid. The images of gels were scanned using a ChemiDoc XRS+ Gel Imaging System (Bio-Rad).
Statistical analysis

Data result from three independent experiments performed in triplicate. The significance of differences were assessed by one-way analysis of variance (ANOVA) using SigmaPlot (Systat Software, San Jose, CA). Pairwise comparisons were analyzed using Tukey’s test. For all tests, a $P < 0.05$ was considered significant.
3.3 Results

3.3.1 The exoproteases were only detected in the biofilm exoproteomes formed at 25°C.

Our preliminary data have shown that *L. monocytogenes* forms biofilms with greater sessile cells and EPS at 37°C than at 25°C. We also found that dispersed cells which were freely living in the supernatant of biofilm cultures were in a greater abundance at 25°C than 37°C. These results indicated that the bacterial populations in different life modes and EPS within biofilms change by environmental temperatures.

To identify exoproteins that are predominant in *L. monocytogenes* biofilms formed in the environment, we compared the proteins in the supernatants of biofilms formed at 25°C and 37°C using 2D-gel electrophoresis. Representative gel images of the exoproteomes formed at 25°C and 37°C are shown in Figure 3.1. Statistical image analysis revealed 15 and 5 protein spots that appeared exclusively in biofilm exoproteomes formed at 25°C and 37°C, respectively.
Proteins were separated by 2-D gel electrophoresis using pH 4-7 linear immobilized pH gradient strips for isoelectric focusing. SDS-polyacrylamide gel electrophoresis was performed with 10% acrylamide gel. 2D-PAGE gels from three independent experiments were aligned to generate reference gels for 25°C (A) and 37°C (B). Gels from two temperatures were compared to determine significant changes in protein spot intensities ($P < 0.05$). Hollow circles indicate the spots present only at one of two temperatures. The spots composed of predicted exoproteins are labeled with the gene IDs listed in Table 3.1.
With LC-MS/MS, total 68 distinct proteins were identified in the temperature-dependent protein spots from the biofilm exoproteomes: 55 from the 15 protein spots exclusively present at 25°C, and 13 from the five protein spots exclusively present at 37°C. Using the Listeriomics database, those identified proteins were assigned to functional groups according to the similarity search of homologous proteins and previous experimental data. The proteins present exclusively at 25°C were generally distributed into three major categories: cell envelope and cellular processes (35%), intermediary metabolism (47%), and others as well as unknown functions (18%). Most (69%) of the proteins present exclusively at 37°C were related to intermediary metabolism (Figure 3.2).
Figure 3.2  Functional distributions of the proteins produced during *L. monocytogenes* biofilm formation at 25°C and 37°C.

The 55 and 13 identified proteins present exclusively in the exoproteomes formed at 25°C and 37°C, respectively, were categorized into functional groups based on the Listeriomics database. The functional groups can be generally grouped into three major categories: cell envelope and cellular processes (left), intermediary metabolism (middle) and others as well as unknown functions (right).
The presence of predicted cell wall/membrane retention signals and specific N-terminal sequences revealed that 14 of the proteins present exclusively at 25°C localize extracellularly and on the cell surface. Furthermore, 12 of those 14 proteins were annotated with functions related to the cell envelope processes such as cell-wall turnover and adhesion (Table 3.1). Further annotation analysis using the MEROPS database revealed that four of the 14 exoproteins detected at 25°C are proteases: two of which (Lmo2505/Spl and Lmo0582/Cwh) are secreted, and the other two of which (Lmo1892/PbpA1 and Lmo0186) are integral to the cell membrane. All four proteases were indicated to function in cell-wall turnover.

Only two of the proteins present exclusively at 37°C (Lmo1847/MntA and Lmo2331) were extracellular/surficial proteins. Of those, only MntA was annotated to function in the cell envelope processes (Table 3.1). Overall, there was a greater number (12) of exoproteins that are associated with cell envelope processes in the biofilm exoproteome at 25°C than that (one) at 37°C.
Table 3.1  Exoproteins in the supernatants obtained from biofilm cultures at 25°C and 37°C.

<table>
<thead>
<tr>
<th>Spot ID</th>
<th>Locus tag</th>
<th>Gene name</th>
<th>Protein accession ID</th>
<th>Functional category a</th>
<th>Description</th>
<th>Theoretical b pI</th>
<th>Experimental pI</th>
<th>MW (kDa)</th>
<th>Unique peptide count</th>
<th>Percentage sequence coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C, Extracellular milieu and cell surface c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7502</td>
<td>lmo0135</td>
<td>ctaP</td>
<td>Q8YAJ0</td>
<td>1.2</td>
<td>ABC transporter, oligopeptide-binding protein, family 5</td>
<td>4.95</td>
<td>58.3</td>
<td>5.8-6</td>
<td>40</td>
<td>11</td>
</tr>
<tr>
<td>8105</td>
<td>lmo0186</td>
<td>spsBl/ yabE</td>
<td>Q8YAE4</td>
<td>1.1</td>
<td>Membrane-bound lytic murein transglycosylase with 3D, G5 and DUF348 domains which belongs to peptidase M23 d</td>
<td>9.00</td>
<td>44.3</td>
<td>6.5-7</td>
<td>20-25</td>
<td>25</td>
</tr>
<tr>
<td>7502</td>
<td>lmo0355</td>
<td>frdA</td>
<td>Q8YA11</td>
<td>1.4</td>
<td>Flavocytochrome c</td>
<td>5.71</td>
<td>46.8</td>
<td>5.8-6</td>
<td>40</td>
<td>6</td>
</tr>
<tr>
<td>1605</td>
<td>lmo0433</td>
<td>inlA</td>
<td>P0DJM0</td>
<td>1.8</td>
<td>Internalin A</td>
<td>4.93</td>
<td>86.5</td>
<td>4.5-5</td>
<td>50</td>
<td>5</td>
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<td>7502</td>
<td>lmo0582</td>
<td>cwh</td>
<td>P21171</td>
<td>1.1</td>
<td>Cell-wall hydrolase/P60 belonging to peptidase C40 d</td>
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<td>50.6</td>
<td>5.8-6</td>
<td>40</td>
<td>13</td>
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<tr>
<td>3504</td>
<td>lmo0927</td>
<td>ltaS</td>
<td>Q8Y8H6</td>
<td>5.2</td>
<td>Putative phosphatidyl-membrane phosphoglyceroltransferase putative LtaS</td>
<td>6.01</td>
<td>74.7</td>
<td>5-5.2</td>
<td>45-50</td>
<td>12</td>
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<tr>
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<td>tcsA</td>
<td>Q48754</td>
<td>1.2</td>
<td>CD4+ T-cell-stimulating antigen</td>
<td>5.02</td>
<td>38.4</td>
<td>5-5.5</td>
<td>35-40</td>
<td>9</td>
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<tr>
<td>3805</td>
<td>lmo1892</td>
<td>pbpA1</td>
<td>Q8Y610</td>
<td>1.1</td>
<td>Penicillin-binding protein group A (S11.001 d)</td>
<td>8.69</td>
<td>90.9</td>
<td>7-8</td>
<td>100-150</td>
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<tr>
<td>3405</td>
<td>lmo2203</td>
<td>flgJ</td>
<td>Q8Y572</td>
<td>1.8</td>
<td>N-acetylmuramoyl-L-alanine amidase</td>
<td>5.00</td>
<td>41.8</td>
<td>5-5.5</td>
<td>35-40</td>
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<tr>
<td>4201</td>
<td>lmo2417</td>
<td>cwh</td>
<td>Q8Y4M0</td>
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<td>ABC transporter, substrate-binding protein</td>
<td>5.27</td>
<td>30.7</td>
<td>5-5.5</td>
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<td>5</td>
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<tr>
<td>7403</td>
<td>lmo2505</td>
<td>spl</td>
<td>Q7AP49</td>
<td>1.1</td>
<td>Secreted protein with lytic activity/P45 as a D-glutamyl-L-m-Dpm peptidase (C40/M23 d)</td>
<td>8.56</td>
<td>42.7</td>
<td>6-6.5</td>
<td>35-40</td>
<td>9</td>
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<td>1204</td>
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<td>Q8Y4D2</td>
<td>3.5.2</td>
<td></td>
<td>Transcriptional regulator LytR</td>
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<td>39.1</td>
<td>4.5-5</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>3504</td>
<td>lmo2526</td>
<td>murA1</td>
<td>Q8Y4C4</td>
<td>1.1</td>
<td>UDP-N-acetylgulosamine 1-carboxyvinyltransferase</td>
<td>4.96</td>
<td>46.0</td>
<td>5-5.2</td>
<td>45-50</td>
<td>6</td>
</tr>
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<td>lmo2569</td>
<td>Q8Y486</td>
<td>1.2</td>
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<td>ABC transporter, dipetide-binding protein</td>
<td>5.25</td>
<td>61.9</td>
<td>5.8-6</td>
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<td>7</td>
</tr>
<tr>
<td>37°C, Extracellular milieu and cell surface c</td>
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<td></td>
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<td></td>
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<td></td>
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<tr>
<td>4311</td>
<td>lmo1847</td>
<td>mntA/ lpeA</td>
<td>Q8Y653</td>
<td>1.2</td>
<td>Manganese-binding lipoprotein MntA</td>
<td>5.43</td>
<td>34.4</td>
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<td>30-35</td>
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<td>2110</td>
<td>lmo2331</td>
<td>Q8Y4U9</td>
<td>4.3</td>
<td></td>
<td>Protein gp23 [Bacteriophage A118]; Phage-related functions</td>
<td>4.89</td>
<td>25.4</td>
<td>4.5-5</td>
<td>20</td>
<td>5</td>
</tr>
</tbody>
</table>

a The identified proteins were assigned to specific functional categories following bioinformatic analysis by similarity searches in the LEGER proteome database. The numbers indicate the functional groups, which are detailed in Table A1 as supplementary materials.

b Theoretical average isoelectric point (pl) and molecular weight (MW) are given for the predicted mature protein without signal peptide.

c The identified proteins were assigned to specific functional categories following bioinformicst analysis by similarity searches in the LEGER proteome database to predict their subcellular localization.

d The peptidases are categorized according to the MEROPS identifier based on the clan and family to which they belong.
3.3.2 Exoprotease genes were expressed at a higher level in sessile cells at 25°C than 37°C

Since a biofilm community generally consists of attached (sessile) and free-living (dispersed) cells, we aimed to explore the contribution of bacterial life modes to the presence of exoproteases. We investigated the expression of the secreted (cwh and spl) and integral membrane (lmo0186 and pbpA1) proteases in sessile and dispersed cells at 25°C and 37°C using real-time PCR. The expression of gene lmo0186, cwh, and spl was significantly greater in sessile cells at 25°C than in sessile cells at 37°C (Figure 3.3); however, the expression of all four genes in dispersed cells was not different between the two incubation temperatures. In addition, the expression of gene lmo0186, cwh, and spl was greater in sessile cells than in dispersed cells at 25°C but not at 37°C.
Figure 3.3  Relative gene expression of identified exoproteases in sessile and dispersed *L. monocytogenes* cells.

Sessile and dispersed cells in biofilms formed at 25°C or 37°C were collected for RNA extraction and qPCR analysis. Cwh (A) and Spl (B) are secreted proteases, while Lmo0186 (C) and PbpA1 (D) are integral membrane proteases. Relative gene expression was calculated by setting the value from the group of sessile cells at 25°C as 1. Data are means ± standard errors from at least three independent experiments with three replicates for each experiment. Different lowercase letters indicate statistically significant differences, $P < 0.05$. 
3.3.3  *L. monocytogenes* biofilm exoproteomes formed at lower temperatures had greater protease activity than those formed at higher temperatures.

Because exoproteases were produced in greater quantities at 25°C than at 37°C, we hypothesized that the proteolytic activity in the biofilm changes in response to environmental temperatures. Gelatin zymography was performed to determine the protease functionality in biofilm exoproteomes formed at 20, 25 or 37°C. Whereas the trypsin positive control did not show a band on the Coomassie Blue gel (Figure 3.4A), it showed a transparent zone on the gelatin gel, representing the proteolytic activity (Figure 3.4B). Only the proteins in the exoproteomes formed at 20°C and 25°C displayed clear transparent zones on the gelatin gels. Additionally, more transparent zones appeared in the exoproteomes formed at 20°C than at 25°C. The transparent zones were located close to the proteins with high molecular weights. The results indicated that the proteolysis was more active in biofilms formed at 20°C and 25°C than in those formed at 37°C.
Figure 3.4  Zymography assay of proteins in the supernatants of biofilm cultures formed at 20°C, 25°C, and 37°C.

Equivalent quantities of proteins (~3.5 mg/ml) isolated from biofilm supernatants at 20°C, 25°C, 37°C; BSA (2 mg/ml); and the protease Trypsin (2.5 µg/ml) as a positive control were loaded onto the gels without (A) or with (B) 0.1% gelatin for SDS-PAGE and enzyme-renaturing incubation. Gels were stained with Coomassie Blue. Three independent experiments were performed, and a set of gels is shown here. The image of the gelatin gel is shown as an inverse phase. The arrowheads and arrow in (B) indicate proteolytic bands of proteins from exoproteomes and trypsin, respectively.
3.3.4 Treatment with protease inhibitor enhanced biofilm biomass only at 25°C.

To test the link between exoproteases and biofilm formation, we applied a protease inhibitor AEBSF to inhibit the functionality of serine proteases during biofilm formation. We found that the AEBSF treatment significantly increased biofilm biomass in a dose-dependent manner at 25°C (Figure 3.5A). By contrast, the AEBSF treatment had no significant effect on the biofilm biomass at 37°C (Figure 3.5B).

Figure 3.5 The effect of protease activity on biofilm formation at 25°C and 37°C.

Bacteria were treated with trypsin and the protease inhibitor AEBSF at the indicated concentrations, following 24 h incubation at 25°C or 37°C. Biofilm biomass was quantified using crystal violet staining and the optical density (O.D.) at 595 nm was measured. The treatment of trypsin with AEBSF represents a positive control for the inhibition and recovery of biofilm formation, which is shown on the left panel. The effect of AEBSF treatment on biofilm formation at 25°C (A) and 37°C (B) is shown on the right panel. Data are means ± standard errors from three independent experiments with at least three replicates for each experiment. * indicates statistically significant differences compared with the negative control group in the same set (\( P < 0.05 \)).
3.4 Discussion

Biofilm-forming ability on various surfaces is a noteworthy character of *L. monocytogenes*. Exoproteins are primary determinants of environmental adaption in *L. monocytogenes* because they mediate the direct interactions between the bacterium and surrounding environments such as food facilities (Lourenço et al., 2013) or the bodies of host (Desvaux, Dumas, Chafsey, Chambon, & Hebraud, 2010; Soni, Nannapaneni, & Tasara, 2011). Most previous works on host infection and intracellular escape have been focusing on the role of exoproteins as virulence factors. However, on a perspective of food safety, understanding the role of exoproteins related to the sessile life mode of *L. monocytogenes* is necessary for the prevention of contamination by this pathogen in food processing environments. We found that *L. monocytogenes* enhances the production of the exoproteases Cwh, Spl and Lmo0186 and proteolytic activity at temperatures typical of those in food processing plants. In addition, our data revealed an inverse relationship between biofilm biomass and the production and activity of exoproteases. The natural break-down of biofilms by endogenous exoproteases is a new facet that should be considered in the development of strategies to control *L. monocytogenes*. Our results provide a basis for further investigations of the molecular mechanisms underlying protease-dependent biofilm dispersion.

The differences in the functions of the exoproteins produced at 25°C and 37°C (Figure 3.2 and Table 3.1) suggest that active cell-wall turnover is one way that *L. monocytogenes* has adapted to the relatively low environmental temperatures outside of host organisms. Furthermore, *L. monocytogenes* produces the exoproteases Cwh, Spl, PbpA1 and Lmo0186 in greater levels when it forms the biofilm at 25°C than it does at 37°C. Consistent with the presence of the proteins, the gene expression of *cwh*, *spl*, and *lmo0186* was greater at 25°C than
at 37°C (Figure 3.3); however, this phenomenon was present only in sessile cells. These results indicate that the gene expression and production of Cwh, Spl, and Lmo0186 are dependent on both the temperature and the bacterial life mode (sessile and dispersed).

Previous studies showed that the expression of cwh, spl, pbpA1 and lmo0186 in planktonic cells was not altered by changes in environmental temperatures, nor was it different between planktonic cells and sessile cells at 37°C (Kaspar et al., 2014; Luo et al., 2013). However, the mRNA level and the protein level of Spl was greater in sessile cells than in planktonic cells at 25°C (Lourenço et al., 2013; Tiong & Muriana, 2016). Based on our results and previous studies, we propose that the expression of certain exoprotease is altered along with changes in the bacterial life mode only within a certain range of temperatures, providing a mechanism to precisely regulate the switch between growth in biofilms and growth within hosts.

In addition to temperature-dependent gene expression and protein production, we found evidence supporting temperature-dependent proteolytic activity in L. monocytogenes. Further experiments using protease-overexpression and deletion strains (Klinkert et al., 2012; Loh et al., 2009) to determine if any temperature-sensing regulators such as non-coding RNAs interact with the exoproteases may shed light on the mechanisms controlling the temperature-dependent switch between bacterial life modes.

The inhibition of protease activity led to an increase in biofilm biomass at 25°C (Figure 3.5), suggesting a negative correlation between the production and activity of exoproteases and the formation of biofilms. In agreement with our results, enhanced biofilm formation was observed in an L. monocytogenes rough isolate with reduced Cwh secretion (Monk, Cook, Monk, & Bremer, 2004). Similar correlations between exoproteases and biofilm development have been demonstrated in other gram-positive organisms. For example, the SigmaB cascade in
Staphylococcus aureus lowers agr RNAIII levels (a quorum sensing system) to reduce the activity of extracellular proteases, resulting in an increment of biofilm formation (Lauderdale et al., 2009). Bacillus subtilis increases exoproteases production as biofilms mature (Marlow et al., 2014). We propose that the presence of exoproteases such as Cwh, Spl, and Lmo0186 contributes to biofilm dispersion in L. monocytogenes. Those exoproteases may also contribute to physiological processes such as the induction of biofilm dispersion, the elimination of damaged cells from the population (Rice & Bayles, 2003), the degradation and recycling of cell-wall components (Siezen et al., 2006; Veening et al., 2008), and the reduction of muramyl dipeptide release, which activates an inflammatory response in the host (Girardin et al., 2003). It will be interesting to identify environmental signals, such as nutrients that are present exclusively either outside the host or inside the host, and molecules which are able to affect the production and activity of exoproteases and thus L. monocytogenes biofilm development.

The production of exoproteases might make biofilms vulnerable to physical disruption from the surrounding environment. As biofilms collapse, L. monocytogenes cells detach from the biofilm and disperse into the surrounding environment, which may lead to contamination in environments where human food is present (Colagiorgi et al., 2017). Therefore, the application of peptidases onto biofilms (Longhi et al., 2008; Nguyen & Burrows, 2014) might aggravate food contamination if the peptidase treatment does not completely kill the bacteria and, in the worst-case scenario, could lead to the dissemination of bacteria with advanced virulence potencies (Guilhen, Forestier, & Balestrino, 2017). On the other hand, a better understanding of the regulatory networks of exoproteins that function on cell wall turnover and the release of cells during L. monocytogenes biofilm development might make it possible to control L. monocytogenes contamination by manipulating physical and environmental conditions.
CHAPTER IV
LINKS BETWEEN S-ADENOSYL METHIONINE AND AGR-BASED QUORUM SENSING FOR BIOFILM DEVELOPMENT IN LISTERIA MONOCYTOGENES

4.1 Introduction

As an environmental pathogen, Listeria monocytogenes replicates and survives both in the environment and within mammalian hosts (Xayarath & Freitag, 2012). Its widespread distribution makes this foodborne pathogen difficult to control and a threat to public health. Such pathogens can survive in the environment by forming surface-associated communities called biofilms (Gutiérrez et al., 2012; Korber, Choi, Wolfaardt, Ingham, & Caldwell, 1997; Poimenidou et al., 2009). Transcriptomic studies recently verified that biofilms comprise heterogeneous populations of bacteria with differences in replication rates and gene regulation between the sessile and planktonic cells (Hamilton et al., 2009; Lazazzera, 2005; Luo et al., 2013). The heterogeneous nature of the biofilm confers the ability to survive under environmental stresses. For example, bacteria within biofilms can alter the expression of genes for higher tolerance to antimicrobial treatments (Chavant et al., 2004; Davies, 2003; Folsom et al., 2010).

Within biofilms, the bacteria are enclosed in self-produced extracellular polymeric substances (EPS), enabling them to sense and adapt to harsh environments (Hall-Stoodley, Costerton, & Stoodley, 2004). EPS represent the three-dimensional scaffold of the biofilm for mechanical stability of biofilms and the adhesion of bacterial cells to surfaces (Flemming &
The production of EPS is closely linked to peptidoglycan synthesis, involving the assembly of saccharide units and peptide bridges by proteins encoded by mur genes and the polymerization of peptidoglycan by penicillin-binding proteins (PBPs) (Typas et al., 2011; van Heijenoort, 2001). Additionally, L. monocytogenes possesses a unique EPS synthesis pathway driven by the Pss complex whose activation requires the c-di-GMP signal modulated by diguanylate cyclases and phosphodiesterases (Chen, L. H. et al., 2014; Köseoğlu et al., 2015).

The coordination of gene expression for biofilm development (Garmyn et al., 2012; Lauderdale et al., 2009), as well as for pathogenicity (Munzenmayer et al., 2016; Riedel et al., 2009) and various cellular functions in bacteria (Grandclément, Tannières, Moréra, Dessaux, & Faure, 2016) has been linked to quorum sensing (QS), a cell-to-cell communication system for the synthesis, secretion and detection of small signal molecules. Two known QS systems have been described in L. monocytogenes. One is encoded by the accessory gene regulator (agr) locus –agrBDCA. This Agr-based QS system (Agr QS) is driven by autoinducing peptide paired with a classical two-component system and plays a role in biofilm formation (Rieu et al., 2007) and pathogenicity (Autret et al., 2003; Riedel et al., 2009). The second QS system based on LuxS is required to produce the signal molecules AI-2 and to synthesize S-Adenosylmethionine (SAM) with the activated methyl cycle (AMC). Although the transcriptional regulation of Agr QS on virulence genes and its own locus have been studied extensively (Garmyn et al., 2012; Pinheiro et al., 2018; Riedel et al., 2009), the transcriptional regulation of the two QS systems in L. monocytogenes on EPS synthesis for biofilms is less clear.

Two groups reported that the amount of AI-2 is reduced and biofilm formation is increased in luxS-deficient mutants (Challan Belval et al., 2006; Sela et al., 2006). However, complementation of the mutant strains with exogenous AI-2 failed to restore the biofilm-forming
ability. A similar failure was also reported in other bacteria (De Keersmaecker et al., 2005; Tannock et al., 2005). Concomitantly, these findings and the absence of AI-2 receptors in many bacteria, including *L. monocytogenes* (Rezzonico & Duffy, 2008), question the role of AI-2 as a QS signal. As LuxS functions as both a synthase for the precursor of AI-2 and an integral enzyme in the AMC, a metabolic role of the AMC in biofilm formation was suggested (Garmyn, Gal, Lemaitre, Hartmann, & Piveteau, 2009) and recently verified in *Streptococcus mutans* (Hu et al., 2018).

Since a metabolite in the AMC, SAM generated from methionine via the synthase MetK is recognized as the methyl group donor for the methylation of macromolecules, polyamine synthesis and SAM radical-mediated vitamin synthesis (Parveen & Cornell, 2011). This indicates that variations in SAM levels might affect a variety of cellular functions. To advance our understanding of the mechanisms underlying *L. monocytogenes* biofilm formation, we investigated the role of the AMC in this process by supplementing bacteria with SAM. Since previously published studies have linked Agr QS to metabolic pathways (Pinheiro et al., 2018; Pohl et al., 2009) and the SAM-binding riboswitch SreA (Loh et al., 2009), we further tested the hypothesis that the SAM-activated methyl cycle (S-AMC) interacts with Agr QS to cooperatively regulate *L. monocytogenes* biofilm formation. Here, we showed that SAM supplement induced biofilm formation under nutrient limitation, revealing a metabolic role of the AMC for *L. monocytogenes* biofilm formation. Notably, we identified the genes in the synthesis of EPS regulated solely by the S-AMC or Agr QS and found these two systems were mutually regulated at the transcriptional level, suggesting redundant regulations on the synthesis of EPS in *L. monocytogenes*. Furthermore, our results indicated that this mutual regulation in bacterial cells was dependent on the transition from the planktonic to sessile life mode.
4.2 Materials and Methods

Bacterial strain and culture conditions

*L. monocytogenes* strain EGD-e (serovar 1/2a) was used in this study, as serovar 1/2a strains account for > 50% of the *L. monocytogenes* isolates recovered from foods and the environment (Aarnisalo et al., 2003; Gilbreth et al., 2005b). The mutants with in-frame deletions of *agrA* (DG125A) and *agrD* (DG119D) were derived from EGD-e and kindly provided by Dr. Pascal Piveteau (Rieu et al., 2007). For all assays, the bacteria were grown in brain heart infusion (BHI) broth (Difco, Sparks, MD) agitatedly for 16 h at 37°C.

Biofilm formation in the presence or absence of S-Adenosylmethionine (SAM)

*L. monocytogenes* (wild type, DG125A, and DG119D) cells were centrifuged, and the pellets were diluted to 10⁷ CFU/ml based on plate enumeration. A 200-μl aliquot of each strain was inoculated into 96-well plates with BHI broth or 10% BHI broth containing 250 and 500 μM membrane-permeable S-(5'-adenosyl)-l-methionine p-toluenesulfonate salt (SAM; Sigma, St. Louis, MO). For RNA extraction from biofilm cultures, a 5-ml aliquot of each strain was inoculated in 6-well plates. The plates were incubated statically at 37°C for 24 h.

Quantitative assay for biofilm formation

The biofilms formed on the surfaces of wells were measured using crystal violet staining as previously described (Lourenço et al., 2012) with minor modifications. Briefly, after the suspension was removed, the wells were air dried and stained with 200 μl of 0.1% crystal violet solution including 20% ethanol for 30 min at room temperature. The unbound dye was removed by rinsing three times with 200 μl sterile double-distilled water, followed by a 30-min air dry. Crystal violet bound to biofilms was solubilized in 200 μl 10% acetic acid with 100 rpm agitation. OD₅₉₅ was measured using a Synergy HT microplate reader (BioTek, Winooski, VT).
RNA extraction and reverse transcription-quantitative PCR (RT-qPCR)

The pellets of sessile cells from biofilm cultures were resuspended in lysis buffer (15 mg/ml lysozyme and 200 µg/ml Proteinase K in TE buffer) and incubated at 37°C for 10 min. The resultant samples were transferred to a lysing matrix B tube (MP Biomedicals, Santa Ana, CA) and vortexed for 15 s for four times using a disruptor (Scientific Industries, Bohemia, NY) with a 1-min pause on ice between vortexes. Total RNA was extracted from the cells using acid phenol-chloroform extraction (Chomczynski & Sacchi, 2006). Five units of RNase-free DNase (Promega, Madison, WI) was applied to the samples at 37°C for 15 min before purification with an RNeasy Plus Universal Mini kit (Qiagen, Germantown, MD). The purity and concentration of RNA were determined by gel electrophoresis and a Nanodrop ND1000 UV-visible light spectrophotometer. One microgram aliquots of RNA samples were reverse-transcribed to cDNA using a SuperScript VILO cDNA synthesis kit (Qiagen). cDNA diluted by a factor of 5, 10, or 20 was used as the template in a 10 µl reaction mixture containing the primers listed in Table 4.1. qPCR was performed with a SYBR green master kit (Applied Biosystem, Foster City, CA) under the following conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s on a 7500 Fast Real-Time PCR system (Applied Biosystems). L. monocytogenes 16S rRNA was used as an internal control. The relative changes in mRNA expression were analyzed by the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Each experiment was repeated at least three times. The significance of the differences among groups was assessed by one-way analysis of variance (ANOVA) using SigmaPlot (Systat Software, San Jose, CA). Pairwise comparisons were performed by using Tukey’s test and the differences were marked by lowercase letters. Student’s t-test was applied to determine a
significant difference (marked by *) between two sets of data. For all tests, a $P$ value of < 0.05 was considered significant.
Table 4.1  Primers used in this study.

<table>
<thead>
<tr>
<th>Name of locus</th>
<th>Locus tag</th>
<th>Primer</th>
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</table>
| agrA          | lmo0051   | F: GAAGATAACAGAATGCAGCGAGAAAGG  
R: GGATCAAACCTCCGAATTTCCCTGAGC |
| agrB          | lmo0048   | F: GCTTATTGATGTTTGTGCTTGCGC  
R: GTGTTCTTCCACCGATTAAGGCAACAC |
| agrC          | lmo0050   | F: GTAGTTTCAGCTTCTATTACGCTTG |  
R: ATACCAACAAATTCCCGCAACATCC |
| agrD          | lmo0049   | F: GAATAAATCAGTTGGTAAATTCTTTCTTAG  
R: CAAATGGACTTTTTGGGATATCAAAAC |
| metK          | lmo1664   | F: TCACTTCTGGGAAGAGATACGTTGTG  
R: CGCATGGTTTAGCTCGAAATTAAC |
|              | lmo2417   | F: ATGCTGGAAGTAGTTAGCGTCTAAG  
R: ATCCAATAACACCCATGCCCAATTAC |
|              | lmo0135   | F: GCAGACTACTCTATCGCACTAAATGG  
R: GATTTCTTGACGTTCTTTGTCGTCAGC |
| murE          | lmo2038   | F: TGTTTCTTGTAAAAGTTAGGCTGTTCG  
R: CGTTAAAAACTCGTTGGGATTACTGGG |
| pbpA1         | lmo1892   | F: AGAGTACACGGAGAAAATGCCTCAATAC  
R: TGGTTTCATAGTAGCCCAACAGAAC |
| dgcA          | lmo1911   | F: CATCTAGTCGAAATGGGGTTTTATC  
R: GAAATAGTAATAACGGAGCCCGGAAG |
4.3 Results

4.3.1 SAM enhanced *L. monocytogenes* biofilm formation

To test the hypothesis that SAM, a two-step precursor of S-Ribosylhomocysteine (SRH) and a methyl-group donor, has an effect on *L. monocytogenes* biofilm formation, we stained biofilms formed in the presence or absence of SAM with crystal violet solution. The biofilm biomass of the wild-type (WT) strain cultured under nutrient limitation (10-fold diluted BHI) was dose-dependently increased with the addition of SAM (Figure 4.1A). The quantified data showed that *L. monocytogenes* biofilm biomass was increased around 1.5-fold in the presence of 500 µM SAM (Figure 4.1B). By contrast, SAM treatment did not significantly enhance the biofilm biomass of the mutants with in-frame-deletion of *agrA* (strain DG125A) or *agrD* (strain DG119D). This indicated that a deficiency in the Agr QS system compromised SAM-enhanced biofilm formation, suggesting a link between intracellular SAM signaling and Agr QS.
Figure 4.1   Visualization and quantification of *L. monocytogenes* biofilm formation in the presence or absence of SAM and the deficient Agr system.

(A) Biofilms were stained by crystal violet solution. (B) The stained biofilm biomass was quantified based on the optical density at 595 nm. Data are means ± standard errors from three independent experiments with three replicates for each experiment. Different lowercase letters indicate statistically significant differences among compared groups, while asterisks (*) indicate significant differences between the two groups pointed out by brackets (*P* < 0.05).
4.3.2 SAM upregulated agr gene expression

To understand whether the SAM-activated methyl cycle (S-AMC) and Agr QS interact with each other at the transcriptional level during biofilm formation, we analyzed the expression of agr genes and genes encoding components of the AMC and amino acid transporters in sessile WT and mutant cells with or without SAM treatment. In the presence of SAM, agrD expression was significantly upregulated, while agrA expression was slightly increased in the sessile WT cells (Figure 4.2A). When comparing WT with two mutants, the expression of metK and lmo2417, responsible for synthesizing SAM and importing methionine, was not noticeably altered in sessile DG125A and DG119D cells (Figure 4.2B). However, the expression of lmo0135, responsible for importing cysteine as another source for SAM production, was greater in sessile DG119D cells than in the WT and DG125A.

Figure 4.2 Regulation of genes associated with Agr QS and the activated methyl cycle during L. monocytogenes biofilm formation in the presence or absence of SAM and the deficient Agr system.

Relative changes in the expression of agrA and agrD in Agr QS (A) and metK, lmo2417 and lmo0135 for synthesizing SAM and importing methionine or cysteine (B) were calculated by setting the value from the group of WT without SAM treatment as 1. Data are means ± standard errors from at least three independent experiments with three replicates for each experiment. Different lowercase letters indicate statistically significant differences among compared groups (P < 0.05).
4.3.3 SAM and Agr QS regulated different pathways of EPS synthesis

Since the addition of SAM induced agr transcription during biofilm formation, we presumed that SAM and Agr QS were both involved in the regulatory network for EPS synthesis. Therefore, we aimed to identify EPS synthesis-associated genes that are regulated by SAM, Agr QS or both. The expression of murE and pbpA1, responsible for the assembly and polymerization of peptidoglycans, increased in the sessile WT cells as the concentration of supplemental SAM increased at the onset of biofilm formation (Figure 4.3A and 4.3B). We further tested the regulation of Agr QS on SAM-dependent expression of murE and pbpA1 in agr mutants treated with SAM. In sessile DG125A and DG119D cells, the treatment of SAM similarly increased murE expression (Figure 4.3A) but not pbpA1 expression (data not shown). Whereas the expression levels of murE and pbpA1 expression were equal among L. monocytogenes WT, DG125A, and DG119D (data not shown), the expression of dgcA encoding a c-di-GMP-producing diguanylate cyclase for Pss complex-driven EPS synthesis was slightly downregulated in the sessile DG125A cells (Figure 4.3C).
Figure 4.3  Genes associated with EPS synthesis were regulated by either SAM or the deficient Agr system.

Relative changes in the expression of *murE* (A) and *pbpA1* (B) for canonical PG synthesis and *dgcA* for Pss complex-driven EPS synthesis (C) were calculated by setting the value from the group of WT without SAM treatment as 1. Data are means ± standard errors from at least three independent experiments with three replicates for each experiment. Different lowercase letters indicate statistically significant differences among compared groups, while an asterisk (*) indicates the significant difference between the two groups pointed out by a bracket ($P < 0.05$).
4.3.4   Agr QS regulated SAM synthesis in the planktonic life mode

Considered that bacterial physiology undergoes a dramatic change during biofilm formation, the gene regulatory networks in the planktonic and sessile life modes were compared to assess the effect of bacterial life mode on the S-AMC and Agr QS. We first investigated the levels of signaling from SAM and Agr QS in the planktonic and sessile WT cells. We found that only the expression of *agrD* was significantly higher in sessile cells than in planktonic cells (Figure 4.4). As the switch from planktonic to sessile life mode affected the level of signaling from Agr QS, we next investigated how this change altered EPS synthesis and the S-AMC. There was no difference in the expression of *murE* and *pbpA1* between planktonic and sessile DG125A or DG119D cells (data not shown). However, the expression of *dgcA* was reduced by the deletion of *agrA* and *agrD* to a greater extent in the planktonic life mode than in the sessile life mode (Figure 4.3 and 4.5A). With regard to the S-AMC, the expression of *metK* and *lmo2417* was significantly upregulated in planktonic DG125A and DG119D cells compared with planktonic WT cells (Figure 4.2 and 4.5B).
Figure 4.4  Expression of genes associated with Agr-based QS, SAM synthesis and transporter of methionine or cysteine in planktonic or sessile *L. monocytogenes* cells.

Relative changes in the expression of *agrA* and *agrD* in Agr QS (A) and *metK*, *lmo2417* and *lmo0135* for synthesizing SAM and importing methionine or cysteine (B) were calculated by setting the value from the group of planktonic WT cells as 1. Data are means ± standard errors from at least three independent experiments with three replicates for each experiment. An asterisk (*) indicates the significant difference between the two groups pointed out by a bracket (*P* < 0.05).

Figure 4.5  Regulation of Agr QS on the genes associated with SAM synthesis, methionine or cysteine transport, and c-di-GMP synthesis.

Relative changes in the expression of *dgcA* for c-di-GMP synthesis (A) and *metK*, *lmo2417* and *lmo0135* for synthesizing SAM and importing methionine or cysteine (B) in planktonic WT, DG125A, and DG119D cells were calculated by setting the value from the group of planktonic WT cells as 1. Data are means ± standard errors from at least three independent experiments with three replicates for each experiment. Different lowercase letters indicate significant differences among compared groups (*P* < 0.05).
4.4 Discussion

The persistence of *L. monocytogenes* and the recurrent cross-contamination of food products are largely attributed to the formation of biofilms on hard-to-clean harborage and their tolerance to environmental stresses (Holch et al., 2013; Lunden et al., 2003). However, the mechanisms underlying these processes are not clear. Researches have begun uncovering the regulation of Agr QS on virulent factors and the autoregulation at *agr* locus (Autret et al., 2003; Garmyn et al., 2012; Paspaliari, Mollerup, Kallipolitis, Ingmer, & Larsen, 2014; Riedel et al., 2009), suggesting that Agr QS orchestrates the pathogenesis and other stress adaptions of *L. monocytogenes* with multiple signal transduction pathways. In this study, we revealed that a regulatory network involving discrepant EPS synthesis pathways is finely tuned by Agr QS and SAM signaling. Critically, our results demonstrate that the signals from Agr QS and S-AMC regulate the transcription of each other’s components, and that this interaction depends on the *L. monocytogenes* life modes (planktonic or sessile). The incorporation of these key activators and clarified mechanisms in the metabolite-oriented QS in current views on the manipulation of *L. monocytogenes* biofilm development will improve strategies to control this foodborne pathogen in food-processing environments.

**SAM signal enhances biofilm formation and agr transcription**

SAM signaling promoted *L. monocytogenes* biofilm formation (Figure 4.1), which is in agreement with the effect of SRH, a SAM-derived product in the AMC, on *L. monocytogenes* attachment (Challan Belval et al., 2006). These pieces of evidence support the metabolic role of AMC in the regulation of *L. monocytogenes* biofilm formation (Garmyn et al., 2009). Since SAM and its binding to riboswitches are involved in the biosynthesis, transport, and utilization of the target metabolite (Loh et al., 2009; Winkler, Nahvi, Sudarsan, Barrick, & Breaker, 2003), it
is conceivable that SAM controls nutrient availability and transduces metabolite-binding events into genetic responses to precisely regulate biofilm formation. We confirmed that the signal from SAM induces the expression of *agr* genes (Figure 4.2A). Currently, regulation of the intrinsic regulator AgrA (Riedel et al., 2009; Rieu et al., 2007) and MouR, a GntR family of transcriptional factor (Pinheiro et al., 2018), are the two known regulatory mechanisms for the transcription of the *agr* locus. Given that *agrD* expression is reduced in a mutant with a deletion of *sreA*, an RNA riboswitch (Loh et al., 2009), it is very likely that SAM-binding SreA is an alternative mechanism contributing to the transcription of the *agr* locus and Agr QS-dependent biofilm formation. Further studies using RNA-RNA gel shifts are needed to characterize the direct interaction between SAM-binding SreA and the *agr* locus. Nevertheless, indirect mechanisms may also contribute to the expression of *agr* genes in response to the SAM signaling, such as via the decay of mRNA by ribonucleases (Baumgardt et al., 2017).

**SAM and Agr QS control different EPS synthesis pathways**

The canonical biosynthesis of peptidoglycan is fundamental for the maintenance of biofilm structures (Freitas, Alves, & Reis, 2011; Rehm, 2010). In addition, a role for the Pss-complex in a c-di-GMP-dependent activation of EPS synthesis in *L. monocytogenes* was recently reported (Chen, L. H. et al., 2014). Our qPCR results indicate that the expression of *murE* and *pbpA1* for peptidoglycan synthesis is regulated by SAM, whereas the expression of *dgcA*, responsible for c-di-GMP synthesis, is regulated by Agr QS (Figure 4.3). These data provide new insights into the mechanisms for precise regulation on EPS synthesis. Specifically, we propose that SAM contributes to EPS synthesis directly by enhancing peptidoglycan assembly and polymerization and indirectly by activating Agr QS for c-di-GMP-dependent EPS production.
Life mode-dependent regulations of Agr QS

Environmental niches and growth phases are crucial determinants of phenotypic heterogeneity in biofilms (van Gestel & Nowak, 2016). In line with the greater abundance of the QS peptide-processing endopeptidase AgrB in attached cells than in planktonic cells (Mata et al., 2015), we found that the expression of agrD was greater in sessile cells compared to their planktonic counterparts (Figure 4.4). This suggests that Agr QS becomes activated with the increase in AgrB and AgrD in sessile L. monocytogenes cells.

Regarding the regulatory network of Agr QS, we found that genes associated with c-di-GMP-dependent EPS synthesis, SAM synthesis and amino acid transport were differently affected by Agr QS according to the bacterial life mode (Figure 4.2, 4.3 and 4.5). In the planktonic life mode, Agr QS inhibits the transcription of components in the AMC and methionine transport, while it enhances the transcription of dgcA for c-di-GMP synthesis. The induction of dgcA expression by Agr QS may contribute to the negative feedback to codY transcription by increasing c-di-GMP synthesis and downregulating GTP concentrations (Elbakush, Miller, & Gomelsky, 2018; Garmyn et al., 2012) and to the activation of Pss complex via increased c-di-GMP (Chen, L. H. et al., 2014). In the sessile life mode, Agr QS inhibits the transcription of components in cysteine transport. It appears that other factors existing in a certain life mode determine the ways that Agr QS regulates the AMC. Moreover, the greater alteration of lmo0135 expression in DG119D relative to that in DG125A implies that the transcription for cysteine transport is more susceptible to the peptide AgrD than the regulator AgrA. Thus, there might be two-component systems other than AgrCA for the detection and transduction of the AgrD signal (Zetzmann, Sánchez-Kopper, Waidmann, Blombach, & Riedel, 2016) or other intracellular regulators requiring AgrD as a cofactor for gene regulation.
A link between metabolism and biofilm formation

Our findings together with those of prior reports provide evidence for the regulation of metabolite-oriented Agr QS during biofilm development. The proposed mechanism includes a metabolic regulator CodY (Bennett et al., 2007; Elbakush et al., 2018; Garmyn et al., 2012; Garmyn et al., 2011) as well as SAM (this study) and its binding riboswitch SreA (Loh et al., 2009) to monitor the nutrient availability and mediate the expression of genes for EPS synthesis (Figure 4.6).
Figure 4.6  Agr-CodY-SAM regulatory network in *L. monocytogenes* under nutrient-rich or -poor conditions.

SAM, CodY, and AgrA are three regulatory factors responsible for the transcription of the *agr* locus. (A) Under nutrient-rich conditions with high concentrations of GTP and SAM, the expression of *agr* genes is upregulated by SAM with the RNA riboswitch SreA (1), CodY binding to GTP (2) and its autoregulation (3). Increased SAM also activates the methyl cycle to induce the transcription of genes for peptidoglycan (PG) synthesis. (B) Under nutrient-poor conditions, the decrease in GTP concentration prevents CodY from being activated, which makes CodY no longer an activator for the *agr* locus. According to planktonic and sessile life modes of the bacteria, Agr QS influences the expression of multiple genes which are responsible for S-AMC, amino acid transport, CodY regulator, and c-di-GMP synthesis. (SAM: S-Adenosylmethionine; SAMC: SAM-activated methyl cycle; PG: peptidoglycan; Agr QS: accessory gene regulator-based quorum sensing, a system including QS peptide precursor AgrD, QS peptide-processing endopeptidase AgrB, kinase receptor AgrC, and response regulator AgrA)
We highlight that S-AMC and Agr QS interact with each other at the transcriptional level and they contribute to EPS synthesis through different routes. Although a limited role of c-di-GMP-induced listerial EPS for biofilm formation (Chen, L. H. et al., 2014), it does not rule out the possibility that a QS-c-di-GMP-EPS pathway is required during the stationary phase for the switch between the planktonic life mode and sessile life mode. A similar pathway with an effect on biofilm dispersion (where bacteria are freely floating again) has been revealed in Vibrio cholera (Waters, Lu, Rabinowitz, & Bassler, 2008) and Xanthomonas campestris (Chin et al., 2010; Tao, Swarup, & Zhang, 2010). Our data also show that Agr QS links to multiple metabolic pathways and that these interconnections are activated in L. monocytogenes only during certain life modes. As metabolic processes such as the metabolism of branched-chain amino acids via CodY and sugar utilization in the phosphotransferase system have been reported to directly and indirectly interact with EPS synthesis and Agr QS (Bennett et al., 2007; Joseph et al., 2008; Lobel & Herskovits, 2016; Pinheiro et al., 2018), further investigation of the role of metabolic regulators such as CodY in Agr QS-associated biofilm formation of L. monocytogenes is warranted.

As S-AMC and Agr QS are cooperative factors in the cross talk between L. monocytogenes methyl metabolism and EPS synthesis, it is suggested that the SAM synthase MetK, SAM-dependent methyltransferases (Zhang & Zheng, 2016), and SAM-mediated peptidoglycan synthesis are potential targets for antagonists (Yadav, Park, Chae, & Song, 2014) combined with Agr QS inhibitors (Fleming & Rumbaugh, 2017; Gray, B., Hall, & Gresham, 2013; Nakayama et al., 2009; Nguyen et al., 2012) to prevent or disrupt listerial biofilms in food-processing environments.
CHAPTER V
CONCLUSION

This work provided evidence supporting a regulatory network controlling *L. monocytogenes* biofilm development with various circuits and at multiple levels from transcription to translation. These revealed molecular mechanisms underlying EPS synthesis also established a starting point for developing biofilm mitigation strategies by targeting components that are responsible for EPS synthesis.

A novel regulatory network consisting of Agr QS, CodY regulators, and SreA riboswitch for biofilm formation is proposed in Figure 5.1A. These signal molecules and relevant regulators allow *L. monocytogenes* to precisely regulate the gene expression for EPS synthesis based on nutrient availability and bacterial life modes. Under nutrient-rich conditions in which SAM and GTP concentrations are in high levels, the *agr* locus is induced by (1) SAM with the riboswitch SreA, (2) CodY binding to GTP and (3) self-encoding AgrA. At the same time, SAM upregulates the gene expression for canonical peptidoglycan synthesis. When *L. monocytogenes* grows into the stationary phase in which environmental nutrients are of shortage, *agr* genes are expressed in a greater level than in the exponential phase. The activated Agr QS cooperates with the activated methyl cycle (AMC) to regulate gene expression for EPS synthesis based on bacterial life modes. This indicates the central role of Agr QS on monitoring nutrient availability for the transition between planktonic and sessile life modes.
Once biofilms mature, *L. monocytogenes* manipulates the expression and production of secreted and integral membrane proteases including Cwh, Spl, and Lm0186 to control the biofilm structure and stability (Figure 5.1B). This naturally occurring break-down of biofilms that leads to biofilm dispersion could enable *L. monocytogenes* to contaminate foods during the food processing.

Overall, these proposed mechanisms for EPS modification decipher how *L. monocytogenes* consolidates and disperses biofilm communities for prolonged survival and dissemination in the environment. To broaden knowledge of *L. monocytogenes* biofilm development and to develop growth- and biofilm-preventing strategies, intriguing future directions include (1) investigating if any temperature-sensing regulators such as noncoding RNAs and transcriptional regulators interact with the exoproteases using protease-overexpression and deletion strains, (2) examining how metabolic-directed regulators such as CodY and SAM-binding RNA elements affect Agr QS, and (3) evaluating the efficacy of antagonists binding to components such as SAM synthase MetK, kinase receptor AgrC and PBPs on the control of EPS synthesis.
Figure 5.1 Life mode-oriented mechanisms underlying biofilm formation and dispersion of *L. monocytogenes*

Planktonic, sessile and dispersed *L. monocytogenes* may simultaneously exist in a biofilm community and unequally contribute to biofilm formation with regulatory networks of SAM signaling, CodY and Agr QS (A) and to biofilm dispersion by regulating the production of exoproteases (B). These bacterial cells in distinct life modes have unique transcriptional patterns, which leads to the phenotypic heterogeneity of biofilm communities. This heterogeneity supports the adaptation and dissemination of *L. monocytogenes* in environments.
REFERENCES


Baumgardt, K., Melior, H., Madhugiri, R., Thalmann, S., Schikora, A., McIntosh, M., . . .


APPENDIX A

THE SUPPLEMENTARY DATA FOR THE STUDY OF EXOPROTEOME IN CHAPTER III
A.1 The detailed functional distributions of the proteins produced during *L. monocytogenes* biofilm formation at 25°C and 37°C.

The table is the supplementary data for the functional distributions of the proteins in Chapter III.

Table A.1 Functional distributions of the proteins produced during *L. monocytogenes* biofilm formation at 25°C and 37°C with details in the names of assigned function category.

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<th>Amounts of proteins at 37°C</th>
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</table>

The identified proteins isolated from biofilm exoproteomes formed at 25°C and 37°C were assigned to specific functional categories following bioinformatic analysis by similarity searches in the LEGER proteome database.
Table A.1 (continued)

<table>
<thead>
<tr>
<th>Group #</th>
<th>Category</th>
<th>Amounts of proteins at 25°C a</th>
<th>Amounts of proteins at 37°C a</th>
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<td>3</td>
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<td>DNA replication</td>
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<tr>
<td>3.2</td>
<td>DNA restriction/modification and repair</td>
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<td>3.3</td>
<td>DNA recombination</td>
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</tr>
<tr>
<td>3.4</td>
<td>DNA packaging and segregation</td>
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<td>0</td>
</tr>
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<td>3.5</td>
<td>RNA synthesis</td>
<td>2</td>
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<td>3.5.1</td>
<td>Initiation</td>
<td>0</td>
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<td>Regulation</td>
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<tr>
<td>3.5.3</td>
<td>Elongation</td>
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<td>0</td>
</tr>
<tr>
<td>3.5.4</td>
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<td>RNA modification</td>
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<td>0</td>
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<td>3.7</td>
<td>Protein synthesis</td>
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<td>Other functions</td>
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<td>Similar to unknown proteins</td>
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<td>From other organisms</td>
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</tr>
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<td>6</td>
<td>No similarity</td>
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</table>

a The identified proteins isolated from biofilm exoproteomes formed at 25°C and 37°C were assigned to specific functional categories following bioinformatic analysis by similarity searches in the LEGER proteome database.
A.2 The intracellular proteins in the supernatants obtained from the biofilm cultures at 25°C and 37°C.

The table reports the intracellular proteins detected in biofilm exoproteomes formed at 25°C and 37°C.

Table A.2 The intracellular proteins present in the supernatant obtained from the biofilm cultures at 25 and 37°C.

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<thead>
<tr>
<th>Spot ID</th>
<th>Locus tag</th>
<th>Gene name</th>
<th>Protein accession ID</th>
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<th>Description</th>
<th>Theoretical b</th>
<th>Experimental</th>
<th>Unique peptide count</th>
<th>Percentage sequence coverage</th>
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<td></td>
<td></td>
<td>pI (kDa)</td>
<td>pI (kDa)</td>
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<td>Uncharacterized protein</td>
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<td>prsI</td>
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<td>Ribose-phosphate pyrophosphokinase</td>
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<td>6-7</td>
<td>30-35</td>
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<td>4201</td>
<td>lmo0396</td>
<td>proC</td>
<td>2.2</td>
<td>Pyrroline-5-carboxylate reductase</td>
<td>5.08</td>
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<td>5-5.5</td>
<td>25</td>
<td>6</td>
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<td>5-5.5</td>
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<td>lmo0689</td>
<td>cheV</td>
<td>1.5</td>
<td>Putative CheA activity-modulating chemotaxis protein</td>
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<td>34.2</td>
<td>4.5-5</td>
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<td>38.0</td>
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<td>35-40</td>
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<tr>
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<td>daph</td>
<td>2.2</td>
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<td>5-5.5</td>
<td>35-40</td>
<td>5</td>
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</tbody>
</table>

a The identified proteins were assigned to specific functional categories following bioinformatic analysis by similarity searches in the LEGER proteome database. The numbers indicate the functional groups, which are detailed in Table A1 as supplementary materials.
b Theoretical average isoelectric point (pI) and molecular weight (MW) are given for the predicted mature protein without signal peptide.
c The identified proteins were screened against the LEGER proteome database to predict their subcellular localization.
d The peptidases are categorized according to the MEROPS identifier based on the clan and family to which they belong.
Table A.2 (continued)

<table>
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<th>Spot ID</th>
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<th>Protein accession ID</th>
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<th>Description</th>
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<th>MW (kDa)</th>
<th>MW (kDa)</th>
<th>Unique peptide count</th>
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<td>ympH</td>
<td>Q8Y797</td>
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<td>M16 family metallopeptidase</td>
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<td>Protein RecA</td>
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<td>53.7</td>
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<td>35-40</td>
<td>10</td>
<td>31.50%</td>
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</tbody>
</table>

a The identified proteins were assigned to specific functional categories following bioinformatic analysis by similarity searches in the LEGER proteome database. The numbers indicate the functional groups, which are detailed in Table A1 as supplementary materials.

b Theoretical average isoelectric point (pI) and molecular weight (MW) are given for the predicted mature protein without signal peptide.

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d The peptidases are categorized according to the MEROPS identifier based on the clan and family to which they belong.
Table A.2 (continued)

<table>
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<th>Gene name</th>
<th>Protein accession ID</th>
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<th>Experimental</th>
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<th>Percentage sequence coverage</th>
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<sup>a</sup> The identified proteins were assigned to specific functional categories following bioinformatic analysis by similarity searches in the LEGER proteome database. The numbers indicate the functional groups, which are detailed in Table A1 as supplementary materials.

<sup>b</sup> Theoretical average isoelectric point (pl) and molecular weight (MW) are given for the predicted mature protein without signal peptide.

<sup>c</sup> The identified proteins were screened against the LEGER proteome database to predict their subcellular localization.

<sup>d</sup> The peptidases are categorized according to the MEROPS identifier based on the clan and family to which they belong.