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GENOME-WIDE SCREENING FOR FUNCTIONAL FACTORS IN LISTERIA MONOCYTOGENES BIOFILM FORMATION

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GENOME-WIDE SCREENING FOR FUNCTIONAL FACTORS IN LISTERIA
MONOCYTOGENES BIOFILM FORMATION

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ABSTRACT

Listeria monocytogenes is an ubiquitous Gram-positive food borne pathogen. Ingestion of *L. monocytogenes* contaminated food can cause serious infections in immune-compromised persons. In addition to planktonic growth, this pathogen can also grow as biofilms under adverse conditions, which has been proved to be more resistant than its planktonic counterpart to various eradications, such as antibiotic treatments. Compared with the extensively studied intracellular replication mechanisms, *L. monocytogenes* biofilm developmental process is not well understood.

Our research group initiated a systemic study on the molecular mechanisms of *L. monocytogenes* biofilm formation. A whole genome-scale screening for functional factors involved in *L. monocytogenes* biofilm development was carried out by means of transposon mutagenesis in combination with microtiter plate assays. 14 mutants with an at least 50% decreased biofilm formation were selected from 10,000 transposon mutants. Transposon locations in these 14 mutants were identified through NEST-PCR and sequencing. The in-frame deletion mutant of two genes, *lmo2553* and *lmo2554*, were generated and showed similar biofilm formation defects as the transposon mutant. The roles of these genes in *L. monocytogenes* biofilm development will be further pursued in the future.

DEDICATION

I would like to dedicate this thesis to my wonderful parents, Lin Ouyang and Meizhen Li, and my husband, Feng Zhou. Thank you Dad and Mom for all your loving support throughout my collegiate pursuits. I will be forever indebted to your example of love and encouragement. And I give deep expression of love and appreciation to Feng for the encouragement you gave and the sacrifices you made during this graduate program.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

ADP   adenosine di-phosphate

ATP   adenosine tri-phosphate

AHL   acylhomoserine lactone

AI-1   autoinducer-1

AI-2   autoinducer-2

BHI   Brain Heart Infusion medium

CHIP   chromatin immune-precipitation

Crp   cAMP receptor protein

CSP   competence signaling peptide

DPD   4,5-dihydroxy-2,3-pentanedione

EPS   extracellular polymeric substance

HTH   helix-turn-helix

HTM   Hsiang-Ning Tsai medium

LB   Luria-Bertani broth

LIPI-1   Listeria Pathogenicity Island 1

LTA   lipoteichoic acid

ORF   open reading frame

PC-PLC   broad range phospholipase C

QS   quorum sensing

SAH   s-adenosyl homocysteine

SOEing   splicing by overlap extension

SRH   s-ribosyl homocysteine

CHAPTER ONE

INTRODUCTION

Listeria monocytogenes, an ubiquitous Gram-positive food borne pathogen, has a wide distribution in the natural environment like water or soil, and is frequently associated with food contamination. As the etiological agent of listeriosis, its intracellular surviving mechanisms, which are primarily governed by the master transcriptional regulator PrfA[1-3], have been relatively well explored. It's been proven by different groups that *L. monocytogenes* is biofilm formation capable as well. As known, biofilm is a special microbial community that attaches to either biotic or abiotic surfaces and grows as a coordinated sessile community. Extracellular polymeric substance (EPS) plays an important role in the maintainance of the complex biofilm structure. Compared with its planktonic counterpart, biofilm is more resistant to various environmental stress, such as antibiotic treatment, nutrient limitation, and physical elimination. This could be a serious problem for those frequently involved in food contamination while at the same time are biofilm-capable, such as *L. monocytogenes*. Since *L. monocytogenes* is a frequent food-associated pathogen, during food processing, it has various access to attach to certain surfaces, for example, plastic containers or stainless steel benches, and develop biofilm. Once occured this will significantly increase the survival of this pathogen and pose a hazardous threatening of food safety.

Currently the developmental process of *L. monocytogenes* biofilm is not well understood. Some factors have been shown to be involved but the detailed functions and

regulatory networks still await elucidation. A better understanding about the molecular mechanisms adopted by this pathogen in biofilm growth will help with the identification of critical factors in this developmental process and potential targets for elimination of biofilm contamination. This could be beneficial for both researches focused on *L. monocytogenes* and industrial issues bothered by biofilm contamination.

This study attempted to address the molecular basis of *L. monocytogenes* biofilm formation by starting with a genome-wide screening for functional factors involved in biofilm development. Transposon mutagenesis was utilized in combination with microtiter plate assays. 14 mutants were selected from a total of 10,000 transposon mutants with an at least 50% decreased biofilm formation. The transposon location in these 14 mutants were identified through NEST-PCR and sequencing. The in-frame deletion mutant of two genes, *lmo2553* and *lmo2554*, were constructed and the biofilm test showed that both have the similar biofilm defect as the transposon mutant.

CHAPTER TWO

LITERATURE REVIEW

Influences of *Listeria monocytogenes* as a food born pathogen

As an ubiquitous Gram-positive bacterium, *L. monocytogenes* exists almost everywhere in our environment. It is such a well-adapted bacterium that it can not only survive vigorously as saprophyte in animal feces or acid mine drainage, but also as intracellular pathogen in various mammalian hosts. It is able to cause serious infections in the newborns, elders, pregnant women and the immune-compromised people. As the etiological agent of listeriosis, it can cause meningoencephalitis, septicemia, placentitis, abortion, neonatal septicemia, febrile gastroenteritis and subclinical pyogranulomatous hepatitis[4]. 2500 cases of listeriosis occur annually in the US, among which 500 cases result in death[5]. *L. monocytogenes* is also a food-borne pathogen that is frequently associated with world-wide outbreaks and is under CDC surveillance. A lot of different types of food can serve as prosperous niches for this pathogen, such as dairy products[6,7], meat[8], and sea food[9]. The contamination of this pathogen in food has always been a concern in the food industry, probably due to its highly evolved ability to survive unfavorable conditions. *L. monocytogenes* can replicate at 1°C[10], which is the refrigeration temperature for most refrigerated food, and it can survive over a pH range from 4.4 to 9.4[10], or in an environment with a water activity as low as 0.92[10]. These characteristics all contribute to its increasing chances to cause human infections.

Contamination by *L. monocytogenes* can become a big economic concern as well, especially for food industry. There was a big recall of imported Manouri Cheese recently due to potential *L. monocytogenes* contamination[11]. Recalls of various products, such as sliced smoked salmon[12] and sprouts[13] were also associated with *L. monocytogenes* contamination. All these recalled products should be destroyed, and this could be a big economic loss for the company.

<div align="center"><i>Listeria monocytogenes</i> PrfA regulon</div>

As a model organism for the study of intracellular pathogenicity, the parasitic mechanisms of *L. monocytogenes* were extensively explored. The development of various genetic tools in the recent 30 years has facilitated the study of pathogenesis of this highly mortal pathogen[14-20]. The availability of genomic sequences of several strains dramatically accelerated the *L. monocytogenes* functional research[21]. *L. monocytogenes* pathogenicity is mainly mediated by the Listeria Pathogeneity Island-1 (LIPI-1) (Figure 2-1), the expression of which is precisely regulated by the master transcriptional factor PrfA[1-3]. The adaptation from a saprophyte to an intracellular pathogen requires the activation of PrfA and the subsequent induction of virulence factors[1,4,22,23].
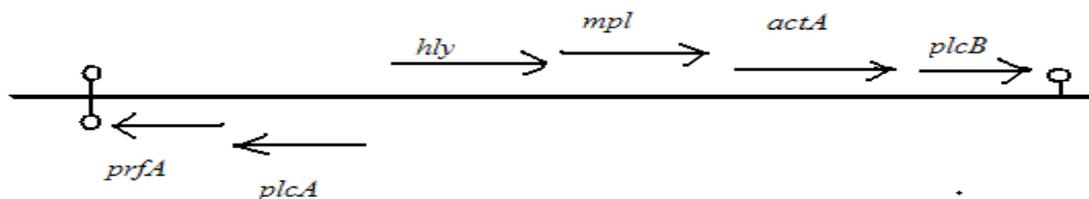
Figure 2-1. Genetic scheme of *L. monocytogenes* Pathogenecity Island-1. The symbol represents the terminator.
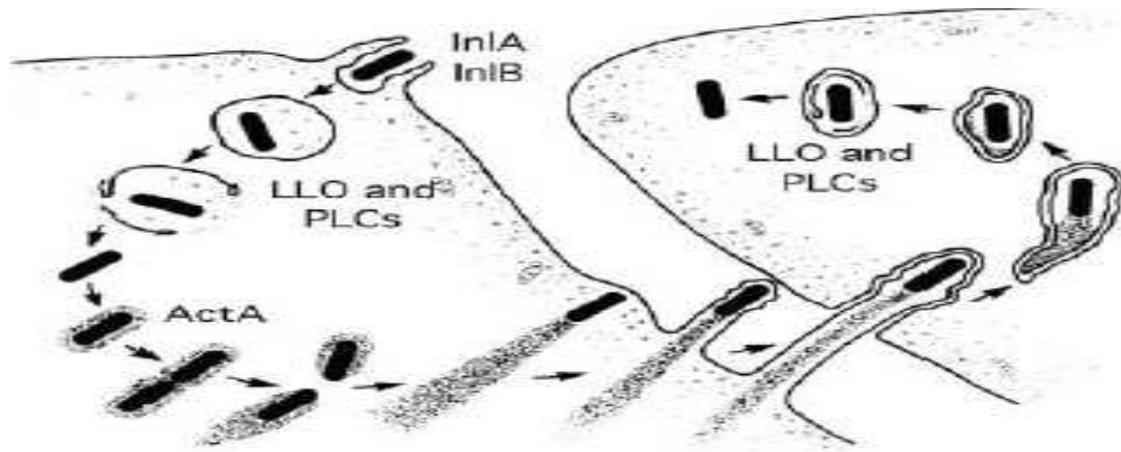
Figure 2-2. Intracellular life cycles of *L. monocytogenes*[28].

As depicted in Figure 2-2, the intracellular life cycles of *L. monocytogenes* can be generally summarized into four stages. The first stage is the invasion of host cells, and for non-professional phagocytic cells this step is mediated by two bacterial surface proteins, internalin A (InlA) and internalin B (InlB)[24,25]. Upon entering the cytoplasm, the bacterium will be entrapped in a membrane-defined compartment called vacuole[4,22]. A successful intracellular infection will require the bacterial escape from the vacuole, which is the second stage of intracellular infection, by the function of bacterial hemolysin Listeriolysin O (LLO) and phospholipases[4]. Then bacterium will proceed to the third stage, which is cytosolic replication using all the nutrients from the cytosol. The fourth stage, cell-to-cell spread is mainly mediated by ActA protein, which plays a central role in the recruitment of host cell actin protein to generate the actin tail and enable the bacterium to move intracellularly[4,26,27]. A double-membrane vacuole is formed upon the invasion of the adjacent cell and the bacterium will repeat another intracellular life

cycle. This direct transmission among the host cells protects the bacterium from encountering the humoral immunity effector cells and molecules and increases the chance of successful infection.

All the aforementioned virulence proteins are members of the PrfA regulon, under control of the positive regulatory factor PrfA, the gene of which is also located on the LIPI-1 downstream of the gene *plcA* (Figure 1-1). This 27 kDa protein belongs to the Crp/Cap regulatory protein family[29] and shares structural similarities with the enterobacterial regulator Crp (cAMP receptor protein). PrfA functions as a homodimer to bind to a palindromic promoter element tTAACanntGTtAa, named PrfA box[1], via the C-terminal helix-turn-helix (HTH) DNA binding motif. The monomer interaction occurs through the N-terminal domain including a β-roll and a long α-helix. Binding of this transcriptional activator will recruit the RNA polymerase to the targeted promoter region and initiate transcription[24]. PrfA was generally considered as essential for *L. monocytogenes* parasitism because *prfA* deletion mutants have been shown to be totally nonpathogenic due to the inability of virulence genes induction[24]. But a recent study on a low-virulence strain A23 did find that even without functional major virulence factors, such as the metalloprotease Mpl, internalin A, internalin B, and phospholipases, the strain could still form plaques and contaminate 100% of inoculate mice[30]. This indicates that this A23 strain might partially keep its virulence by some unidentified mutations[30].

PrfA regulation is well manipulated by *L. monocytogenes* at transcriptional, post-transcriptional, and post translational levels[24]. Regulation at post translational level

mainly occurs through the changes of PrfA binding activity[1]. PrfA in the saprophytes normally exists in a native form which is weakly active, while binding to the unknown cofactor in the host cell will turn PrfA into the fully activated state[1]. Lots of study about various PrfA* mutants, such as Gly145Ser[31,32], Ile45Ser[33], Gly155Ser[34], Leu140Phe[35] and Glu77Lys[34], do prove that by mutation of specific amino acid outside of the HTH motif, PrfA can be locked on the highly active conformation and induce the PrfA-dependent virulence expression to a similar high level observed in the intracellular infection. In contrast, mutation inside the HTH motif significantly decreases the DNA binding affinity and leads to a loss of virulence[36]. In addition to this positive activating pathway, negative regulatory pathways have been found as well. One obvious evidence is the activated charcoal effect. It was well observed that adding activated charcoal to the culture medium can dramatically stimulate the virulence genes expression[37], and the adsorption of an unknown diffusible autorepressor by the activated charcoal was suspected to mediate this phenomenon. The other evidence of negative regulation is the observation that expression of virulence genes are downregulated in glucose or fructose supplemented medium even in the presence of activated charcoal[38-40]. This might suggest independent pathways for the sugar-mediated regulation and autorepressor-mediated regulation.

It was well proven that *prfA* can be transcribed as both monocistronic and bicistronic[42,43]. Two promoters, P*prfA*P1 and P*prfA*P2, as shown in Figure 2-3, exist in the intergenic region between *prfA* and *plcA*. P*prfA*P1 is a σ^A-dependent promoter which is responsible for the low level PrfA synthesis in the normal environmental setting

bacteria[1], while P$prfA$P2 can be regulated by both σ^A and σ^B and mainly mediates the elevation of virulence expression under stress conditions[1,44]. The 5' untranslated region (UTR) of transcripts generated from P$prfA$P1 promoter can form a secondary structure at low temperature such as 30°C to work as a thermosensor[45]. This secondary structure will only melt at high temperature, for example 37°C which is the normal human body temperature, and reveal the ribosome binding site at the 5' UTR for PrfA translation. This might partially explain the rapid PrfA-dependent virulence induction upon host infection. $prfA$ can also be transcribed as a bicistronic transcript from the P$_{plcA}$ (Figure 2-3), and since P$_{plcA}$ is PrfA-dependent, it has a positive feedback on PrfA synthesis.
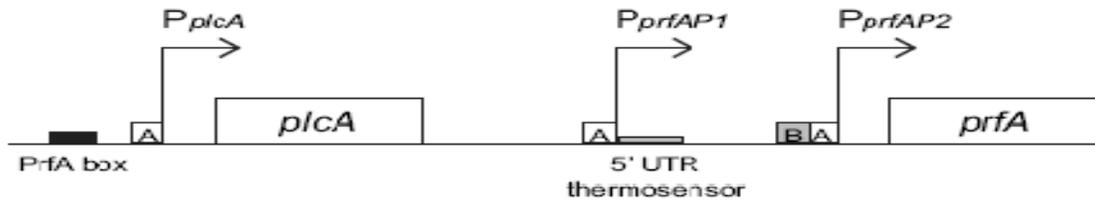
Figure 2-3. The organization of the three promoters that control the $prfA$ transcription[41]. Square labeled A represents the recognition site for σ^A factor, and square labeled B represents the recognition site for σ^B factor.

Distinctive PrfA binding affinity to different promoters could be another way to manipulate PrfA regulation. More PrfA are required for activation of those promoters with mismatches in the PrfA box than those exactly matched promoters[46,47]. And the RNA polymerase (RNAP) binding affinity of the promoters could also plays a role in the expression regulation of the PrfA-dependent virulence genes[1].

As the master regulator of *L. monocytogenes*, PrfA was considered as the critical regulator that mediates the transition from the extracellular free-living motile life style to the intracellular pathogen. But a very recent study from Kolter's group just provided solid evidence for the idea that PrfA also contributes to biofilm formation, and is involved in the transition from the extracellular free-living style to the biofilm style[41]. *prfA* mutants were defective in biofilm formation after initial surface adhesion[41], and the PrfA conformational change for biofilm formation is different from those required for intracellular virulence expression[41]. It would be interesting to investigate the molecular mechanisms of PrfA biofilm promotion.

<u>Bacterial biofilms</u>

Biofilm is generally considered as an aggregate of microbial cells that attaches to either biotic or abiotic surfaces and grows as a matrix-encased community. Naturally occurred biofilm usually consists of mixed species populations, and this provides the growing community two advantages, a reciprocal balance in the whole community when the nutritional condition is altered due to the different physiological metabolism properties of the mixed population [48], and increased overall biofilm fitness due to the cooperation between these species through their various properties in cellular attachment, matrix synthesis, dispersal, motility and toxin production[48]. Both Gram-negative and Gram-positive bacteria, including *L. monocytogenes,* have been shown to be capable of biofilm growth on the surfaces of various materials.

Through detailed study of biofilm development of various bacteria, a general five-step developmental process has been proposed to govern the most bacterial biofilm formation[49].The first step is the initial attachment of single cells, which usually is reversible and followed by the second step of irreversible attachment and beginning of extracellular polymeric substance (EPS), or the so-called matrix production. The mechanisms and mediators of the cell attachment have been a focus of biofilm research for a long time, and one good example is the role of flagellum. Flagellum was generally considered as required for initial cell attachment in *E. coli*[50], *P. aeruginosa*[51], and *L. monocytogenes*[52,53], although evidences of its inhibitory effect on cellular attachment were also found by other group[54], and whether it serves as a adhesion molecule or motility factor is still under debation. As the matrix production continues and the matrix accumulates, the biofilm architecture begins to establish in the third step, and then become mature in the fourth step. The matrix plays an important role in the maintenance of the biofilm structure[55]. Proteins, exopolysaccharides, lipids, and nucleic acids have been identified as components of the extracellular matrix while their relative contribution to the whole community might vary among different species[49]. In *L. monocytogenes*, proteins[56] and extracellular nucleic[57] acids were identified to be two components of its biofilm matrix. Due to the importance of matrix in biofilm, enzymes such as glycosidases, proteases, or deoxyribonucleases that can degrade biofilm matrix have been proposed as potential methods to control or eliminate biofilm growth[58]. Studies with scanning confocal laser microscopy (SCLM) revealed that generally the sessile bacteria grow in matrix encased clusters which are separated by a network of open water channels

for the purpose of nutrient exchange[49]. While some species biofilms exhibit a mushroom-like 3D structure[49], *L. monocytogenes* biofilm shows a different type that consists a network of knitted chains[59]. Finally, cell dispersion is shown to be an important and regulated step in the homeostasis of the biofilm community[49]. It was recently reported that in *P. aeruginosa*, as the biofilm structure becomes mature, matrix-free cavity would be formed inside the clusters and swimming cells will be released from this cavity at the dispersion stage[60]. This self-active dispersal process called seeding[58], is one of the three distinct modes of biofilm dispersion[58], the other two types including erosion, which usually refers to continuous release of small clusters of cells from the biofilm, and sloughing, which defines the sudden detachment of large portions of the biofilm[58].

Biofilm has been proposed as an integral phase of bacterial life cycle which is usually adopted when the bacteria confront unfavorable living conditions. It's been extensively demonstrated that compared with its planktonic counterpart, biofilm is much more resistant to the environmental stress, such as nutritional limitation, antibiotic[61], detergent treatment[62], or organic acids treatment[63]. In addition to functioning as the penetration obstacle attributing to its complex architecture[49], the strategies adopted by this sessile group for increased antibiotic resistance have been revealed to include several conventional mechanisms, for example, chromosomal β-lactamase, up-regulated efflux pumps, and mutations in the antibiotic target molecules[64].

Transcriptional regulation in biofilm development

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In *Bacillus subtilis*, the most thoroughly studied Gram-positive organism and also the close relative of *L. monocytogenes*, several transcriptional factors (Figure 2-4) have been revealed to affect the bacterial biofilm formation, especially in terms of the matrix production, through the precise regulation of their downstream genes expression. The main matrix components of *B. subtilis* biofilm are encoded by two operons, the 15-gene *eps* operon which encodes the enzymes involved in exopolysaccharides production[65], and the *yqxM-sipW-tasA* operon[66] which encodes the secreted matrix protein TasA. The *eps* operon and the *tasA* operon are both repressed by SinR[67], the master regulator for *B. subtilis* biofilm, and this repression is released by the interaction of SinR with the anti-repressor SinI[68]. While under normal conditions SinR is constitutively expressed in the bacterial community, SinI is under the positive control of Spo0A bistable switch[68]. Two other regulators, AbrB and Sigma H factor, are also involved in *B. subtilis* biofilm transcriptional regulation. AbrB works as a negative regulator for biofilm formation by repressing the expression of one putative secreted protein YoaW and the signal peptidase SipW[69], while AbrB expression is under the direct negative control of Spo0A [70]. In contrast, Sigma H factor is suggested to indirectly stimulate the biofilm formation by activation of Spo0A expression[69,71]. A recent interesting observation found that expression of the anti-repressor SinI will be turned on only in a subpopulation of the *B. subtilis* biofilm community, thus the derepression of *yqxM-sipW-tasA* operon and *eps* operon in this subpopulation actually provides matrix for the whole biofilm community[68]. This labor-division system enables the *B. subtilis* cells to incorporate various environmental signals, such as nutritional or stress signals, to coordinate various

physiological processes, such as sporulation, cannibalism and biofilm formation[72], through this complex regulatory network and provide itself the best survival strategy.
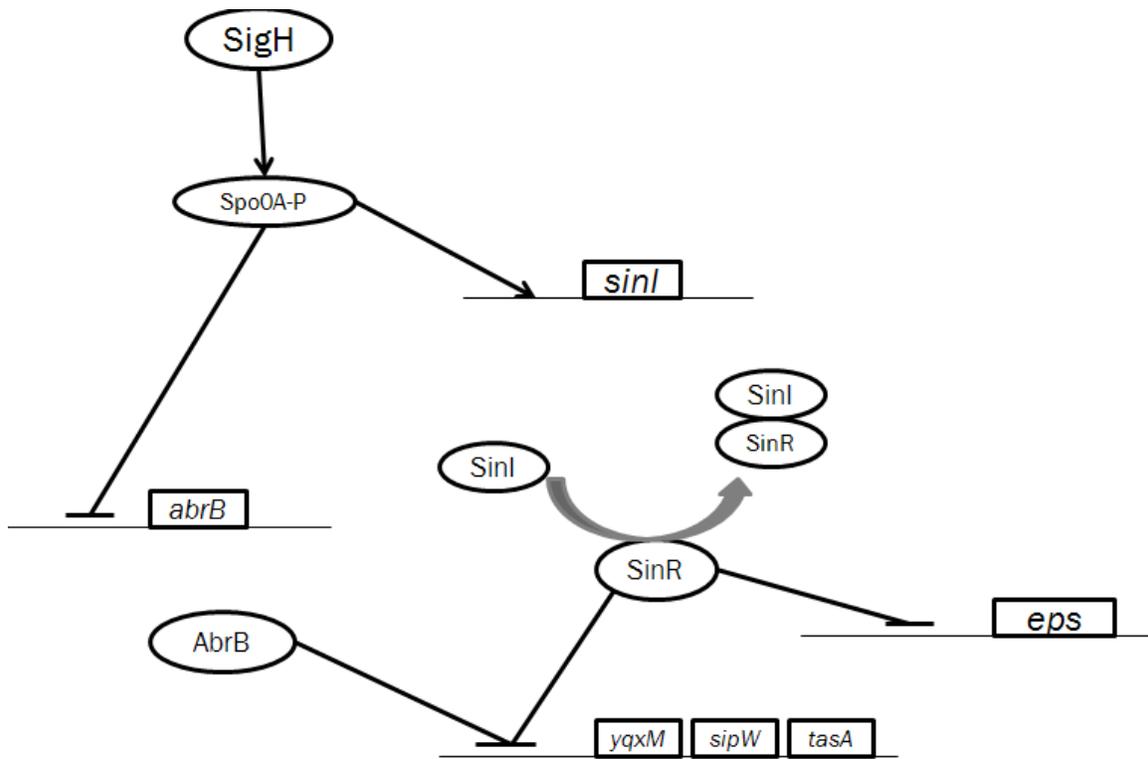
Figure 2-4. The transcriptional regulation network in *B. subtilis* biofilm.

In *L. monocytogenes*, currently only one transcriptional regulator, DegU, has been directly proven to mediate biofilm formation. *L. monocytogenes* DegU is an orphan response regulator which binds to its own promoter and works as an auto-represser[73]. It also binds to the promoter of *motB* operon and positively regulates the expression of GmaR[73], which is the anti-repressor of flagellar synthesis. DegU plays a role in bacterial motility, chemotaxis, virulence and biofilm of several Gram positive

species[73,74]. Deletion of DegU led to reduced *L. monocytogenes* biofilm formation[73] and its indirect regulation on flagella was suggested to mediate its role in biofilm[75]. Considering the involvement of flagella in *L. monocytogenes* biofilm[52], it would be tempting to look at if some other factors, such as MogR which has been shown to regulate flagellum motility[76] and virulence[77], also has a role in biofilm formation. Further study on biofilm matrix might provide more hints to unravel the transcriptional regulation network of *L. monocytogenes* biofilm.

Quorum sensing in biofilm

Quorum sensing (QS) generally refers to the bacterial inter-species or intra-species communication based on the population-dependent production and secretion of certain diffusible small compounds. The accumulation of these signal molecules would be detected when it reaches a threshold, and stimulates the cellular responses in forms of regulated gene expression and coordinated population behavior. Quorum sensing has been found in both Gram-positive and Gram-negative bacteria. Different types of molecules function as the "language", and this communication actually mediates various physiological processes including bacterial pathogenesis[78], bacteriocin production[79], competency development[80], biofilm formation[81,82] and multidrug resistance[83,84].

In the Gram-positive *Streptococcus pneumonia*, the broadly studied competence signaling peptide (CSP) QS system was demonstrated to influence the biofilm growth[82]. CSP is an oligopeptide product of the *com* regulon. *comA* and *comB* encode the secretion apparatus for CSP, while *comC* encodes the CSP precursor. The accumulated CSP is

detected by ComD, a surface histidine kinase receptor for CSP, and the signal is transmitted through the response regulator ComE, which induces the *com* regulon[85]. The subsequent product ComX is a sigma factor that regulates the expression of several groups of downstream genes[86]. Initially the CSP QS was found to affect bacterial competence. Accumulation of CSP to the threshold stimulates a subpopulation of the bacterial community to lyse and release nucleic acid, which is taken up by the remaining cells[87]. Addition of CSP increases the DNA level in the matrix as well as the biofilm growth[88,89]. Considering the role of nucleic acid as a component of biofilm matrix, it was suspected that the effects of CSP on biofilm growth could be related with its induction of cell lysis and DNA release[90]. But the details of the coordination of these two distinct yet correlated bacterial population behaviors still await elucidation.

Acylhomoserine lactone (AHL), also known as autoinducer-1 (AI-1)[91], is one QS signal that is only found in the Gram-negative microorganisms currently. LuxI and LuxR mediate this AHL QS system. LuxI is the AHL synthase while LuxR is the cytoplasmic receptor for AHL and at the same time the transcriptional activator for downstream target genes[92]. When the cell density is low, AHL production level is low as well and not enough AHL can diffuse into the cytoplasm and bind to LuxR, thus the unstable LuxR will be degraded. Only when the cell density reaches a certain high level, AHL will be accumulated to a concentration high enough to bind to LuxR and stabilize as well as activate the downstream gene expression[92]. It was recently found that among the many operons affected by AHL, one is the *rhlAB* which controls rhamnolipids production[93]. Changes of rhamnolipids production by rhlAB mutation resulted in

dramatically altered biofilm structure from a mushroom-like to a flat, undifferentiated one[94]. This AHL QS system controls the bacterial colonization of eukaryotes[95], and is critical for successful interaction of *Pseudomonas aeruginosa* with the animal or plant tissues[96]. Absence of AHL results in decreased biofilm formation in *Pseudomonas aeruginosa*[97] and *Yersinia pestis*[91], and the homologous QS system in *Pantoea stewartii* was proven to affect biofilm formation by controlling the exopolysaccharide production and the cell adhesion[92].

Another broadly studied QS signal is the Autoinducer 2 (AI-2) that exists in both the Gram-positive and Gram-negative microorganisms[98-101]. AI-2 was initially discovered in *Vibrio harveyi* as the quorum sensing molecule to regulate bioluminescence[102]. AI-2 refers to the collection of cyclic derivatives of 4,5-dihydroxy-2,3-pentanedione (DPD), which is a highly reactive metabolic by-product of the activated methyl cycle[103]. DPD production depends on two catalytic enzymes, Pfs and LuxS[104]. Pfs catalyzes the conversion of s-adenosyl homocysteine (SAH) into s-ribosyl homocysteine (SRH), and LuxS catalyzes the conversion of SRH into DPD[104]. While this *luxS*-dependent AI-2 QS system has a negative regulation on the biofilm formation of *Bacillus cereus* and *Staphylococcus epidermidis*[98,99], it was reported to positively regulate *Streptococcus mutans* biofilm formation[105]. It also mediates the increase of *Streptococcus anginosus* biofilm in the presence of sub-MICs of antibiotics[103], and *Streptococcus gordonii* biofilm formation ability in a mixed-species environment with *Porphyromonas gingivalis*[106]. In *L. monocytogenes* biofilm, LuxS seems to play a negative role because deletion of LuxS leads to a denser biofilm[104,107].

But addition of AI-2 molecules couldn't restore the normal biofilm level. Instead, addition of the SRH was able to modify the biofilm growth[104]. The role of AI-2 in *L. monocytogenes* was suggested to limit to detoxification of SAH, and might be irrelevant to QS[104].

In *L. monocytogenes*, one quorum sensing system that has been shown to be involved in biofilm formation is the *agr* system. This system is encoded by a four-gene operon which contains a two-component regulatory system by coding the histidine kinase AgrC and response regulator AgrA, a signal peptide AgrD and the enzyme AgrB involved in AgrD processing[108]. High level of signal peptide AgrD due to increased cellular population enables AgrD to bind to the histidine kinase AgrC, which activates the response regulator AgrA by phosphorylation[109]. Activated AgrA then turns on the regulation of the downstream genes, which currently are unclear in *L. monocytogenes*. The *agr* system plays a role in both *S. aureus*[110] and *L. monocytogenes*[108,111] biofilm. Deletion of the signal peptide AgrD or response regulator AgrA resulted in decreased biofilm, and the *agr* operon expression level change appeared during the biofilm development did not happen to the planktonic growth[108].

Considering the broad involvement of QS in biofilm formation, QS has been studied for its potential as the biofilm eradication target. Inhibitors and antagonists of the QS were evaluated for their possible effects and consequences on the whole community as well as individual cell[112].

CHAPTER THREE

MATERIALS AND METHODS

Strains, plasmids, oligonucleiotides and growth conditions

Strains, plasmids and oligonucleotides used in this study are listed in Table 3-1, 3-2 and 3-3. *E. coli* was cultured in Luria-Bertani broth (LB) (Bacto, USA) at 37°C with vigorous shaking unless otherwise specified. Planktonic *L. monocytogenes* was cultured in Brain Heart Infusion medium (BHI) (Bacto, USA) at 37°C with vigorous shaking unless otherwise specified. *L. monocytogenes* biofilm were grown in LB medium or Hsiang-Ning Tsai medium (HTM) [113] at 37°C without shaking unless otherwise specified. In *E. coli*, 100 µg/ml ampicillin was used to select for ampicillin resistance, 25 µg/ml chloramphenicol was used to select for chloramphenicol resistance, and 30 µg/ml kanamycin was used to select for kanamycin resistance. In *L. monocytogenes*, 200 µg/ml streptomycin was used to select for streptomycin resistance, 5 µg/ml erythromycin was used to select for erythromycin resistance, and 10 µg/ml chloramphenicol was used to select for chloramphenicol resistance.

Determination of biofilm growth conditions

Bacterial overnight cultures were grown in 200 µl BHI medium in 96-well polystyrene plates at 37°C without shaking, then 5 µl of the overnight culture was transferred to 200 µl HTM or LB medium in 96-well polystyrene plates to grow biofilm. Biofilm cultures were incubated at either 37°C or 30°C without shaking for 24 h, 48 h or

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72 h. For the biofilm test, the supernatant cultures were discarded and each well was washed with 250 µl PBS for 5 times. 210 µl of 1% crystal violet was added to each well to stain the attached cells for 1 h, followed by distill water wash for 5 times. Then 220 µl of 95% ethanol was added to each well and incubated at room temperature for 6 h. The absorbance of the ethanol solution at 570 nm was measured using the MULTISCAN EX plate reader (Thermo, PA, USA).

Transposon mutagenesis

The competent cells of *L. monocytogenes* were prepared as follows. A 10 ml overnight culture of *L. monocytogenes* strain 10403S was grown in BHI at 37°C with vigorous shaking. Then 3 ml of the overnight culture was inoculated into 100 ml sucrose/BHI. The freshly inoculated culture was grown at 37°C with vigorous shaking until the OD.600 reaches 0.2. Then 100 µl penicillin G (10 mg/ml) was added to the culture, and the incubation was continued for 2 h more. Then the bacterial culture was centrifuged at 7000 rpm for 10 min at 4°C. After decanting the supernatant, the cell pellet was washed 3 times with the ice cold wash solution (1 mM HEPES/0.5M sucrose), once with 100 ml and twice with 50 ml. Finally the cells were resuspended in 250 µl of the ice cold wash solution (1 mM HEPES/0.5M sucrose), and flash frozen in 100 µl aliquots at -80°C.

The transposon mutagenesis was performed as follows. 0.2 µg transposon plasmid pMC38[114] was used for the electrophoration of 100 µl 10403S competent cells at 1.8 KV, 400Ω, 25µFad. The electroporated cells were recovered in 2 ml BHI (0.5M sucrose)

medium at 30°C for 2 h, and then were selected on BHI agar plates with 200 µg/ml streptomycin and 5 µg/ml erythromycin at 30°C for 48 h. Transformants on the plates were inoculated in 2 ml BHI medium with 5 µg/ml erythromycin and 10 µg/ml kanamycin and incubated at 30°C overnight. 50 µl of the overnight culture was transferred to a 10 ml BHI medium with 200 µg/ml streptomycin and 5 µg/ml erythromycin and the incubation was continued at 30°C for 2 h, and then the incubation temperature was shifted to 42°C to lose the plasmid on purpose and the incubation continued for 6 h. 100 µl of the 1:100 diluted culture was directly plated on BHI agar plates with 200 µg/ml streptomycin and 5 µg/ml erythromycin, and the transposon mutants on these plates were grown at 42°C overnight.

Microtiter plate assay

The transposon mutants were grown in 200 µl BHI medium with 200 µg/ml streptomycin and 5 µg/ml erythromycin in 96-well polystyrene plates at 37°C overnight without shaking, and 5 µl of the overnight culture was transferred to 200 µl HTM medium in 96-well polystyrene plates for biofilm growth at 37°C for 48 h without shaking. For the biofilm test, the supernatant culture was discarded and each well was washed with 250 µl PBS for 5 times. 210 µl of 1% crystal violet was added to each well to stain the attached cells for 1 h, followed by distill water wash for 5 times. Then 220 µl of 95% ethanol was added to each well and incubated at room temperature for 6 h. The absorbance of the ethanol solution at 570 nm was measured using the MULTISCAN EX plate reader (Thermo, PA, USA).

Linkage test

U153 phage [115] was used for the transduction. First of all, transposon mutants were grown in LB medium with 200 µg/ml streptomycin and 5 µg/ml erythromycin at 30°C until the OD.600 reached 0.2. U153 phage stock was diluted to 10^6~10^4 titration, and 100 µl of diluted phage solution was mixed with 100 µl transposon mutants culture and incubated at room temperature for 40 min. Then 3 ml LB soft agar (0.75% agar) was mixed with each phage-bacterium mixture and poured onto LB agar plates with 10 mM $CaCl_2$ and 10 mM $MgSO_4$, following by incubation at room temperature for 24 h.

The plaque layer of the LB agar plates was soaked in 1 ml TM buffer (10 mM Tris HCl pH7.5, 10 mM $MgSO_4$) for 25 min, then a sterile spreader was used to screw up the plaque layer, and both the TM solution and pieces of plaque layer were collected in a sterile centrifuge tube. The mixture was vortexed vigorously, and then centrifuged at 8000 rpm for 10 min. The supernatant was transferred to another sterile centrifuge tube, mixed with 1/10 Volume of chloroform, and kept at room temperature for 10 min after vigorous vortex. The supernatant was collected as the mutant phage stock.

100 µl of mutant phage stock was mixed with 200 µl *L. monocytogenes* 10403S culture which was grown in LB medium at 30°C, and incubated at room temperature for 1 h. Then 3 ml BHI soft agar (0.75%) was mixed with the mixture and poured onto BHI agar plates with 10 mM sodium citrate and 5 µg/ml erythromycin. Plates were incubated

at 37°C for 2 h, and then another layer of BHI soft agar was added to the plates. The incubation was continued at 37°C for 48 h. Colonies growing on the plates were randomly selected for the biofilm test as previously described, and the biofilms of these colonies were compared with the wild type 10403S and the original transposon mutants.

Probe preparation and southern blot

The probe for erythromycin resistance cassette in *mariner* transposon was amplified using pMC38 plasmid as the template and primer # 88 and # 89. The resulting PCR fragment was purified by QIAGEN QIAEX II purification kit. The purified product was labeled with Biotin 3' End DNA Labeling Kit (Pierce, IL, USA, PROD # 89818) as follow: First of all 100 ng DNA probe was denatured by heating in boiling water for 5 min. Then 50 µl of reaction mixture, which contained 10 µl of 5x TdT Reaction Buffer, 5 pmol final 3'-OH end of pre-denatured DNA probe, 5 µl of 5µM Biotin-11-UTP, 5 µl of 2 U/µl diluted TdT and ultrapure water, was prepared by adding individual components to the same reaction tube. The mixture was incubated at 37°C for 30 min, and the reaction was stopped by adding 2.5 µl of 0.2M EDTA. The labeled DNA probe was purified with chloroform: isoamyl alcohol, heated at 100°C for 5 min and placed on ice for the subsequent hybridization.

The genomic DNA of the *L. monocytogenes* transposon mutants were prepared with Wizard genomic DNA isolation kit (Promega, WI, USA), and 5µg of genomic DNA was digested with Hind III (TaKaRa, Japan) at 37°C overnight. Then the digested DNAs were applied to 0.8% agarose gel and electrophoresed at 25 constant voltage for 10 hours.

After electrophoresis, the gel was acid-depurinated by 0.25N HCl and denatured with 0.5M NaOH and 1.5M NaCl for 45min, then neutralized with 0.5M Tris and 1.5M NaCl for 45min. DNA was transferred to positively charged nylon membrane (GE, Canada, VCAT# NP0HYB0010) using a downward transfer apparatus. After transfering, DNA was cross-linked to the membrane using the UVP CL-1000 Ultraviolet Crosslinker (254nm, autosetting) (UVP, CA), and the membrane was incubated in pre-hybridization buffer (6x SSC, 5x Denhardt solution, 0.5% SDS, 100μg/ml denatured fish sperm DNA) at 55°C for 2 h. Then hybridization was performed using hybridization buffer (6x SSC, 0.5% SDS) with labeled DNA probe (30 ng per ml of hybridization buffer) at 55°C overnight. On the next day, the membrane was washed 3 times for 15 min per wash with gentle agitation using the 50°C preheated wash buffer (2x SSC, 0.1% SDS). Then the signal was detected using Chemiluminescent Detection Kit (Pierce, IL, USA, PROD # 89880) according to the provided protocol.

Transposon localization

Arbitrary-Primed PCR, as described before[116], was utilized to identify the location of the transposon on the chromosome. First round PCR was performed using primer # 5 paired with # 7 or # 10 in a final volume of 25μl with fresh colony from BHI plates and Choice Taq Polymerase (Denville, NJ, USA), and PCR was performed under following conditions: 95°C for 2min; 25 cycles of 94°C for 30sec, 42°C for 45sec, 72°C for 1min; and a final extension at 72°C for 5min. Then 5μl of the 1:25 dilution of the first round PCR product was used as the template for the second round PCR. The second

round PCR was performed using primer # 6 paired with # 8 or # 11 in a final volume of 25µl with Choice Taq Polymerase (Denville, NJ, USA) under the following conditions: 95°C for 2min; 25 cycles of 94°C for 30sec, 45°C for 45sec, 72°C for 1min; and a final extension at 72°C for 5min. The amplified products were subjected to agarose gel (1%) electrophoresis. Fragments from the agarose gel were excised and purified by QiaEXII (Qiagen, Germany). The purified fragments were sent for sequencing (Genomic Institute, Clemson University) using primer # 9 or # 12. The obtained sequences were blasted using NCBI nucleotide blast program (http://blast.ncbi.nlm.nih.gov/BlastoCgi).

Construction of in-frame deletion mutant

Various sets of primers (#37~#40 for *lmo1256*; #41~#44 for *lmo2553*; #45~#48 for *lmo2554*; #49~#52 for *lmo1083*) were used to amplify upstream fragment and downstream fragment of target genes using genomic DNA of 10403S as the template with High Fidelity Taq Polymerase (Roche, Switzerland) in a final volume of 25 µl. Then 1 µl of upstream and downtream fragments were mixed and used as template for the Gene Splicing by Overlap Extension (gene SOEing)[117] as described before to generate in-frame deletion fragment. The amplified product was subjected to agarose gel (0.8%) electrophoresis, and purified by QiaEXII (Qiagen, Germany). The purified product was digested with EcoR I and Hind III restriction enzymes (TaKaRa, Japan). After purification with the QiaEXII (QIAGEN, Germany), the digested fragment was ligated with the pKSV7 plasmid[19], which was digested with the same restriction enzymes and

purified. *E.coli* DH5α competent cells were transformed with the ligation products and selected for ampicillin resistance on LB plates.

High purity pKSV7-deleted fragment plasmids were isolated from the *E. coli* transformants using PureYield Plasmid Miniprep System (Promega, WI, USA). Electroporation was performed to transform the pKSV7-deleted fragment plasmids into 10403S competent cells at 1.8 KV, 400 Ω and 25 µFad. Transformants were selected for chloramphenicol resistance on BHI agar plates. The integrants were selected by incubation at 42°C in BHI plates supplemented with 10µg/ml chloramphenicol. The plasmid containing the wild type copy of the corresponding gene after alleic exchange was removed by passaging the bacterial culture in the BHI medium without chloramphenicol at 30°C for 8~12 passages. The bacterial colonies spread on BHI plates were randomly selected and tested for chloramphenicol sensitivity. Chloramphenicol sensitive colonies were subjected to PCR using respective primers to confirm the deletion.

Table 3-1. Strains used in this study.

Strains	Genotype	References
E.coli		
DH5α	F⁻ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–	lab stock
CE49	pKSV7-Δ*1083* in DH5α	this study
CE51	pKSV7-Δ*2553* in DH5α	this study
CE52	pKSV7-Δ*2554* in DH5α	this study
CE53	pKSV7-Δ*1256* in DH5α	this study
L.monocytogenes		
10403S	a streptomycin resistant isolate of strain 10403, 1/2a serotype	lab stock
NF-L943	PrfA G155S mutation in 10403S background with *actA-gus-plc*B transcriptional fusion	28
TM-1	transposon insertion at *lmo0644* in NF-L943 background	this study
TM-2	transposon insertion at *lmo1262*/*lmo1263* in NF-L943 background	this study
TM-6	transposon insertion at *lmo2553*/*lmo2554* in NF-L943 background	this study
TM-16	transposon insertion at *lmo0707* in NF-L943 background	this study
TMY-95	transposon insertion at *lmo2205* in 10403S background	this study
TMY-169	transposon insertion at *lmo0734* in 10403S background	this study
TMY-235	transposon insertion at *lmo0106* in 10403S background	this study
TMY-386	transposon insertion at *lmo0086* in 10403S background	this study
TMY-408	transposon insertion at *lmo2534* in 10403S background	this study
TMY-423	transposon insertion at *lmo2535* in 10403S background	this study
TMY-438	transposon insertion at *lmo2529* in 10403S background	this study
TMY-486	transposon insertion at *lmo0676* in 10403S background	this study
TMY-489	transposon insertion at *lmo1370* in 10403S background	this study
TMY-521	transposon insertion at *lmo2229* in 10403S background	this study
CL-9	Δ*plcA* and Δ*plcB* in 10403S	118
CL-10	Δ*hly* in 10403S	119
CL-18	Δ*inlB* in 10403S	120
CL-19	Δ*inlA* in 10403S	120
CL-25	Δ*mpl* in 10403S	121
CL-35	Δ*actA* in 10403S	122
CL-57	Δ*prfA* in 10403S	123
CL-59	Δ*lmo1083* in 10403S	this study
CL-60	Δ*lmo1256* in 10403S	this study
CL-61	Δ*lmo2554* in 10403S	this study
CL-62	Δ*lmo2553* in 10403S	this study
CL-63	Δ*lmo2553* in 10403S	this study
CL-64	Δ*flaA* in 10403S	124

Table 3-2. Plasmids used in this study.

Plasmids	Description	References
pMC38	mariner transposon vector with Bsu PmrgA, ts ori	108
pKSV7	intergrational vector derived from pE194ts	14
pMC43	in-frame deletion of *lmo1083* in pKSV7	this study
pMC45	in-frame deletion of *lmo2553* in pKSV7	this study
pMC46	in-frame deletion of *lmo2554* in pKSV7	this study
pMC47	in-frame deletion of *lmo1256* in pKSV7	this study

#	Name	Sequences	Applications	References
1	Marq112	5'-CGC CAG GGT TTT CCC AGT CAC GAC-3'	FOR CHECKING AND SEQUENCING CLONES IN pKSV7	14
2	Marq113	5'-AGC GGA TAA CAA TTT CAC ACA GGA-3'	FOR CHECKING AND SEQUENCING CLONES IN pKSV7	14
5	ARB1	5'-GGC CAC GCG TCG ACT AGT ACN NNN NNN NNN GTA AT-3'	ARBITRARY PRIMER 1 FOR NESTED PCR	108
6	ARB2	5'-GGC CAC GCG TCG ACT AGT AC-3'	ARBITRARY PRIMER 2 FOR NESTED PCR	108
7	Marq 255	5'-CAG TAC AAT CTG CTC TGA TGC CGC-3'	MARINER LEFT END PRIMER FOR 1ST ROUND NESTED PCR	108
8	Marq 256	5'-TAG TTA AGC CAG CCC CGA CAC CCG-3'	MARINER LEFT END PRIMER FOR 2ND ROUND NESTED PCR	108
9	Marq 257	5'-CTT ACA GAC AAG CTG TGA CCG TCT-3'	MARINER LEFT END PRIMER FOR SEQUENCING	108
10	Marq 269	5'-GCT CTG ATA AAT ATG AAC ATG ATG-3'	MARINER RIGHT END PRIMER FOR 1ST ROUND NESTED PCR	108
11	Marq 270	5'-TGT GAA ATA CCG CAC AGA TGC GAA-3'	MARINER RIGHT END PRIMER FOR 2ND ROUND NESTED PCR	108
12	Marq 271	5'-GGG AAT CAT TTG AAG GTT GGT ACT-3'	MARINER RIGHT END PRIMER FOR SEQUENCING	108
37	1256 up forward	5'-G**GA ATT C**TT AGC ATC TAC TTT GGC ATC C-3'	FOR GENERATION OF lmo1256 IN FRAME DELETION	this study
38	1256 up reverse	5'-GTA TTT TTA TGC TGT TTA TTG TTT CAT GCC CAT CTC TCC-3'	FOR GENERATION OF lmo1256 IN FRAME DELETION	this study
39	1256 down forward	5'-GGA GAG ATG GGC ATG AAA CAA TAA ACA GCA TAA AAA TAC-3'	FOR GENERATION OF lmo1256 IN FRAME DELETION	this study
40	1256 down reverse	5'-CCC **AAG CTT** AAA AAT ACC GTA ACA AAG AGG-3'	FOR GENERATION OF lmo1256 IN FRAME DELETION	this study
41	2553 up forward	5'-G**GA ATT C**TG GGC AGG CGG ATT TTC TTT TGG T-3'	FOR GENERATION OF lmo2553 IN FRAME DELETION	this study
42	2553 up reverse	5'-GAT TTT TTG GGA CGC TGT ATC TAC CTT TCA CTC CTT CGT TAA-3'	FOR GENERATION OF lmo2553 IN FRAME DELETION	this study
43	2553 down forward	5'-TTA ACG AAG GAG TGA AAG GTA GAT ACA GCG TCC CAA AAA ATC-3'	FOR GENERATION OF lmo2553 IN FRAME DELETION	this study
44	2553 down reverse	5'-AA**C TGC AG**C CTT CCA AGC ATA GCA CCC ATT AAA TA-3'	FOR GENERATION OF lmo2553 IN FRAME DELETION	this study
45	2554 up forward	5'-G**GA ATT C**GG GAT CTG TTT GTG AGT GC-3'	FOR GENERATION OF lmo2554 IN FRAME DELETION	this study
46	2554 up reverse	5'-GCC TAA CCA TAT TTC AGC ACC CTG ACC TTT TAC TTT TTC-3'	FOR GENERATION OF lmo2554 IN FRAME DELETION	this study
47	2554 down forward	5'-GAA AAA GTA AAA GGT CAG GGT GCT GAA ATA TGG TTA GGC-3'	FOR GENERATION OF lmo2554 IN FRAME DELETION	this study
48	2554 down reverse	5'-CCC **AAG CTT** ACT AAA AAG TTC AGC ACG ACC-3'	FOR GENERATION OF lmo2554 IN FRAME DELETION	this study
49	1083 up forward	5'-GC**T CTA GA**A TAA CCA CTC TCT TTC TGT G-3'	FOR GENERATION OF lmo1083 IN FRAME DELETION	this study
50	1083 up reverse	5'-GAT AGT TTC TTT AAT TCC TGT ATA AGT CAG CAA ATC TAA GTT TAC-3'	FOR GENERATION OF lmo1083 IN FRAME DELETION	this study
51	1083 down forward	5'-GTA AAC TTA GAT TTG CTG ACT TAT ACA GGA ATT AAA GAA ACT ATC-3'	FOR GENERATION OF lmo1083 IN FRAME DELETION	this study
52	1083 down reverse	5'-GG**G GTA CC**T TTT TTT CTA AAG CAA CT-3'	FOR GENERATION OF lmo1083 IN FRAME DELETION	this study
88	Marq254	5' - CGTGGAATACGGGTTTGCTAAAAG - 3'	INTERNAL OF ermC FOR AMPLIFICATION OF PROBE FOR SOUTHERN BLOT	108
89	Marq206	5' - TGTCAGACATATGGGCACACGAAAAACAAGT - 3'	3'-END OF ermC FOR AMPLIFICATION OF PROBE FOR SOUTHERN BLOT	108

CHAPTER FOUR

RESULTS

Optimization of biofilm growth conditions

In order to select the proper condition which can stimulate robust biofilm growth, various combinations of medium and temperature were tested for their ability to promote *L. monocytogenes* biofilm growth before the screening. After testing the wild type 10403S biofilm growth at 37°C and 30°C in either LB or HTM medium, 37°C and HTM, minimal medium were finally selected as the standard conditions for the subsequent microtiter plate assays, because bacteria had the most robust biofilm growth under these conditions. As shown in Figure 4-1, regardless of the temperature, HTM medium promotes better biofilm growth than LB medium, while 37°C is better than 30°C no matter which medium was used.

Selection of biofilm abnormal mutants

A total of 10,000 mutants were generated through transposon mutagenesis. The biofilm formation ability of these mutants were compared with the wild type 10403S strain and the *flaA* in-frame deletion mutant in HTM, the minimal medium in 96-well polystyrene plates. Under our test conditions, the OD.570 of the 10403S biofilm after 48 h incubation generally was around 1.40, while the OD.570 of *flaA* mutant was around 0.70. 14 mutants were selected for at least 50% decrease biofilm formation compared with the 10403S, and biofilm formation of 4 mutants were shown in Figure 4-2. The

growth rates of these 14 mutants were studied in both BHI and HTM medium, and none of them showed any growth defect compared with the wild type 10403S. Southernblot revealed the single transposon insertion in these mutants, and linkage test showed that the biofilm defects of these mutants indeed linked with the transposon insertion.

Identification of transposon locations

By arbitrary-primed PCR and sequence analysis in NCBI Nucleotide data base, the transposon location in these 14 mutants were successfully identified. In some mutants the transposon inserted in the open reading frame of genes, while in others it located between adjacent open reading frames. The transposon location in 3 mutants were shown in Figure 4-3,4-4, 4-5 and the descriptions of these genes were summarized in Table 4-1.

The role of *lmo1083, lmo1256, lmo2553 & lmo2554* in biofilm formation

In Tm-6 mutant, the transposon located at the end of the open reading frame of the gene *lmo2554* between 916 bp and 917 bp. Because there are only 6 bp between the open reading frame of the gene *lmo2554* and *lmo2553*, this transposon insertion probably would affect the transcription of both *lmo2554* and *lmo2553* since these two genes are normally co-transcribed. Thus in-frame deletion mutant of both genes were constructed, and tested for biofilm growth in polystyrene tubes. In HTM medium, a reproducible defect compared with the wild type was observed among all the three time points tested with Δ*lmo2554* strain (Figure 4-7), with a 87% decrease at 24 h, a 96% decrease at 48 h and 72 h. Biofilm of Δ*lmo2553* strain decreased as well, with a 84% reduction at 24 h, a

75% reduction at 48 h and 72 h (Figure 4-7). To collect the biofilm growth information from different medium, biofilm growth of constructed deletion mutants were also tested in LB medium. In LB medium biofilm of Δ*lmo2554* strain had a 70% decrease at 24 h and 72 h, and a 53% decrease at 48 h compared with the wild type (Figure 4-6), while biofilm of the Δ*lmo2553* strain had about a 45% reduction at 24 h, 48 h and 72 h (Figure 4-6).

In addition to these two, in-frame deletion mutant of other two genes, *lmo1083* encoding a protein similar to dTDP-D-glucose 4,6-dehydratase, and *lmo1256* encoding a hypothetical protein, were also constructed. These two genes were identified from other project. Biofilm test of these two mutants were performed with HTM and LB medium. Interestingly Δ*lmo1083* strain had a similar biofilm growth pattern as the wild type in HTM medium (Figure 4-7), while in LB medium the absence of *lmo1083* led to a 77% decrease at 24 h, a 37% decrease at 48 h and a 51% decrease at 72 h (Figure 4-6). Absence of *lmo1256* did not affect the biofilm formation in LB or HTM medium. Thus *lmo1083* might also play a role in *L. monocytogenes* biofilm formation, and this effect might relate to the specific medium used.

<u>PrfA and virulence genes in biofilm</u>

Biofilm of the PrfA mutant and other virulence genes mutants were also compared with the wild type strain 10403S in LB and HTM medium. As shown in Figure 4-8, the absence of PrfA and other virulence genes had a minimal effect on biofilm growth in LB. But in HTM medium (Figure 4-9), deletion of PrfA led to a 70% biofilm reduction at 24

h, and a 40% reduction at both 48 h and 72 h. Most of the tested virulence mutants had a similar biofilm level as the wild type, except for the *plcA⁻ plcB⁻* double mutant and *mpl⁻* mutant (Figure 4-9). Absence of *plcA* and *plcB* resulted in a similar 40% biofilm reduction as that of the *prfA⁻* mutant at 48 h and 72 h, but only minimal decrease was observed at 24 h. A 50%, 66%, and 80% decrease at 24 h, 48 h and 72 h respectively was observed for the *mpl⁻* mutant.
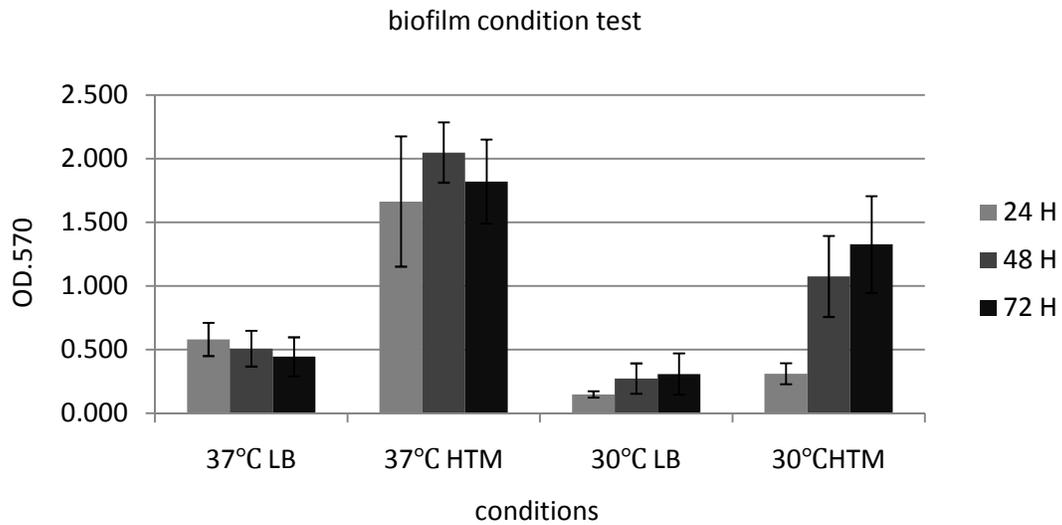
Figure 4-1. Average biofilm growth of the wild type 10403S strain from 3 parallel repeats in LB and HTM medium in 96-well polystyrene plates at 37°C and 30°C after different incubation periods.
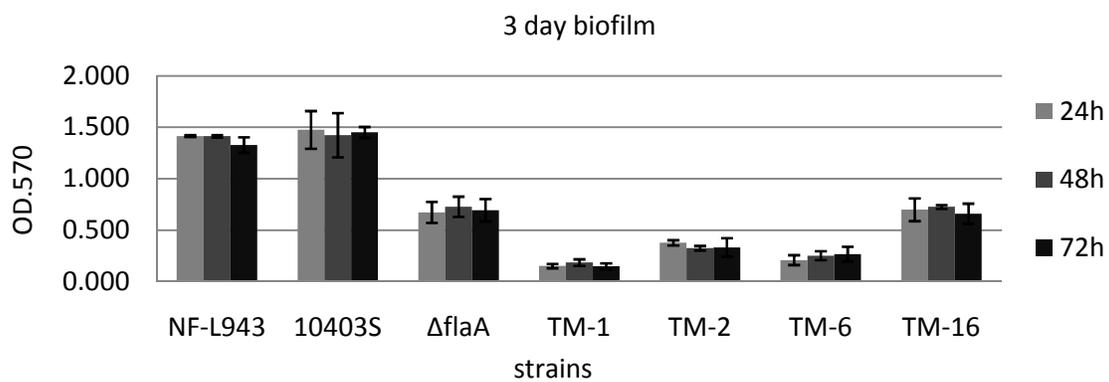
Figure 4-2. Average 3-day biofilm formation of several transposon mutants from 3 parallel repeats in HTM medium in 96-well polystyrene plates.

Figure 4-3. Transposon location in mutant TM-2, which led to the identification of the genes *lmo1262* and *lmo1263*. The symbol ⌀ represents the terminator. The symbol ⌐ represents the location of the transposon, and the direction represents the orientation of the erythromycin resistance cassette in the transposon.
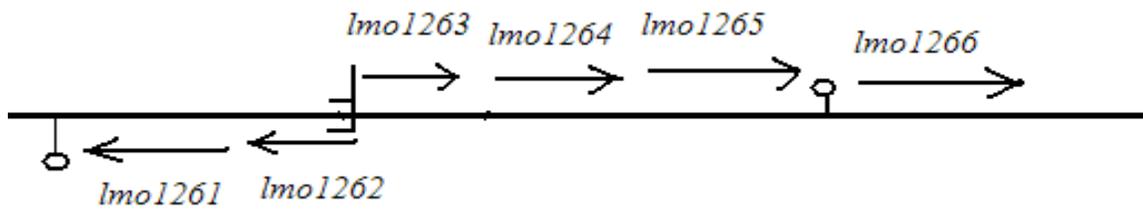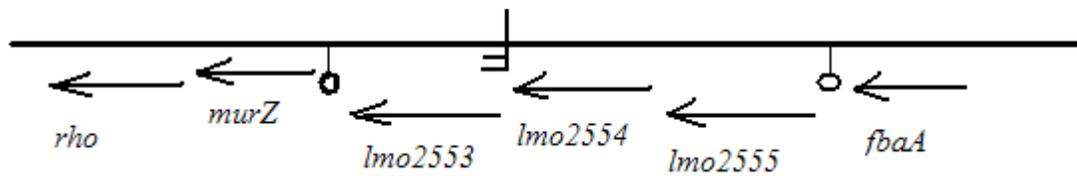
Figure 4-4. Transposon location in mutant TM-6, which led to the identification of the genes *lmo2553* and *lmo2554*. The symbol ⦵ represents the terminator. The symbol ⊐ represents the location of the transposon, and the direction represents the orientation of the erythromycin resistance cassette in the transposon.

Figure 4-5. Transposon location in mutant TM-16, which led to the identification of the gene *lmo0707*. The symbol ⍲ represents the terminator. The symbol ⊢ represents the location of the transposon, and the direction represents the orientation of the erythromycin resistance cassette in the transposon.
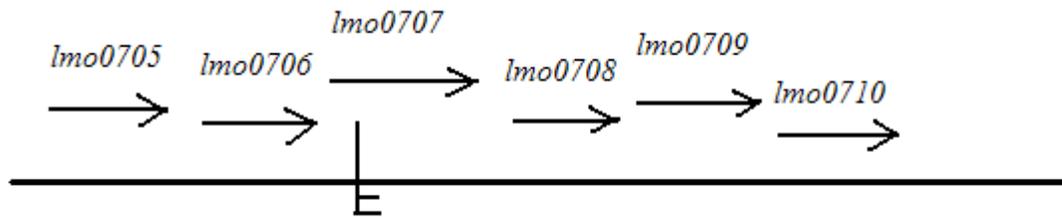
Table 4-1. Identified genes through transposon localization in this study.

Gene	Description	48 h Defect
lmo0734	Similar to transcriptional regulator (LacI family)	3.1
lmo1262	similar to transcriptional regulator(phage-related)	4
lmo1263	similar to transcriptional regulator	4
lmo2553	hypothetical protein	5.6
lmo2554	similar to galactosyltransferase	5.6
lmo0106	DltD protein for D-alanine esterification of lipoteichoic	7.6
lmo0644	LTA synthesis, Transfer initial glycerolphosphate to form GroP-Gal-Glc-DAG	6.8
lmo1370	Similar to branched chain fatty acid kinase	50
lmo2229	Similar to penicillian- binding protein	5.4
lmo2205	Similar to phosphoglyceromutase1	3.6
lmo0086	Phosphoribosylformylglycinamidine synthetase I	17.9
lmo2534	AtpE	12.2
lmo2535	AtpB	14.9
lmo2529	AtpD	4.4
lmo0685	Flagella biosysthesis protein	4.8
lmo0707	Flagellar FliD	2

biofilm in LB,tube

Figure 4-6. Average biofilm formation of the in-frame deletion mutants from 3 parallel repeats in LB medium in polystyrene tubes compared with the wild type 10403s and Δ*flaA* strain.

biofilm in HTM,tube

Figure 4-7. Average biofilm formation of the in-frame deletion mutants from 3 parallel repeats in HTM medium in polystyrene tubes compared with the wild type 10403s and Δ*flaA* strain.

Figure 4-8. Average biofilm formation of the PrfA mutant and virulence mutants from 3 parallel repeats in LB medium in polystyrene tubes compared with the wild type 10403s strain.

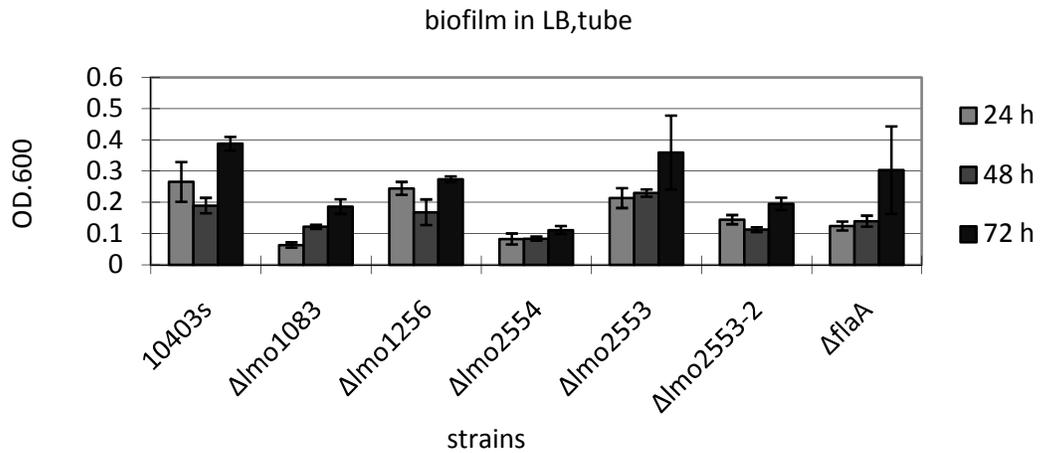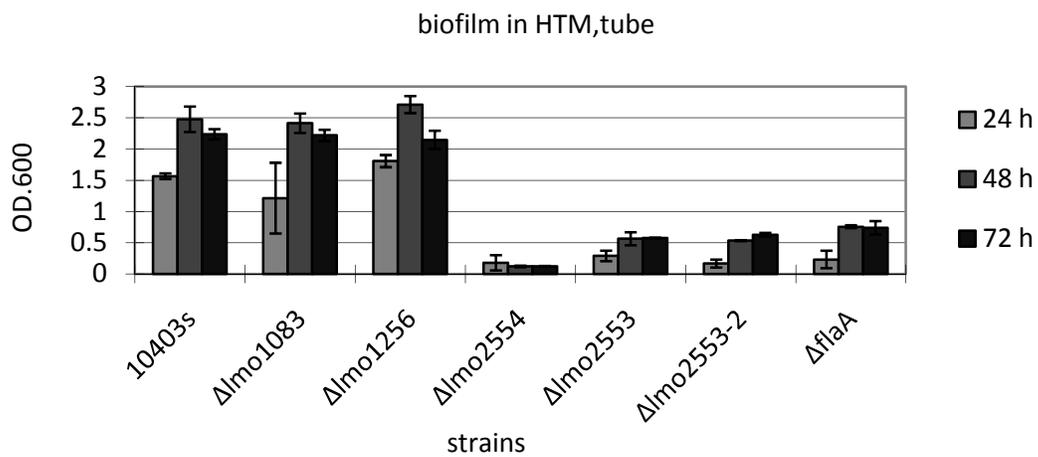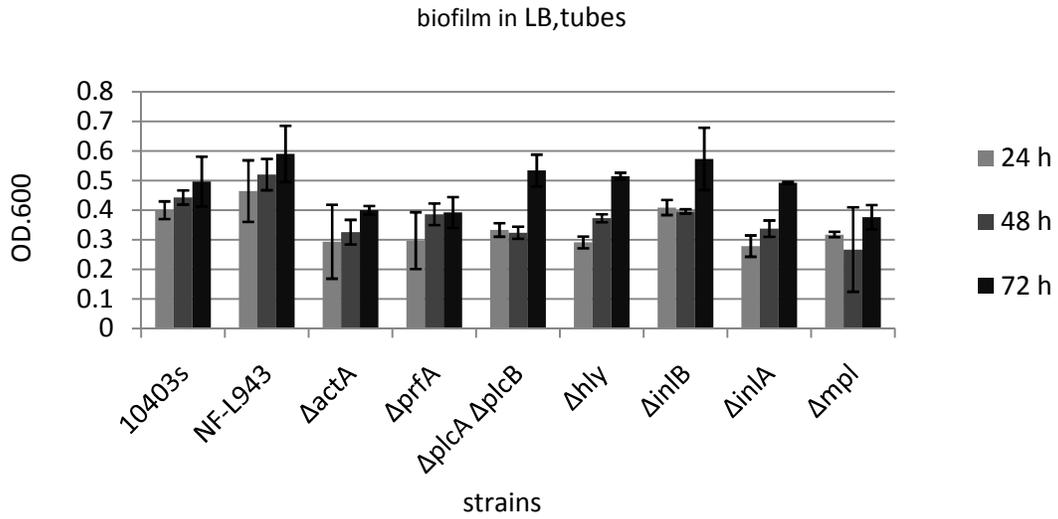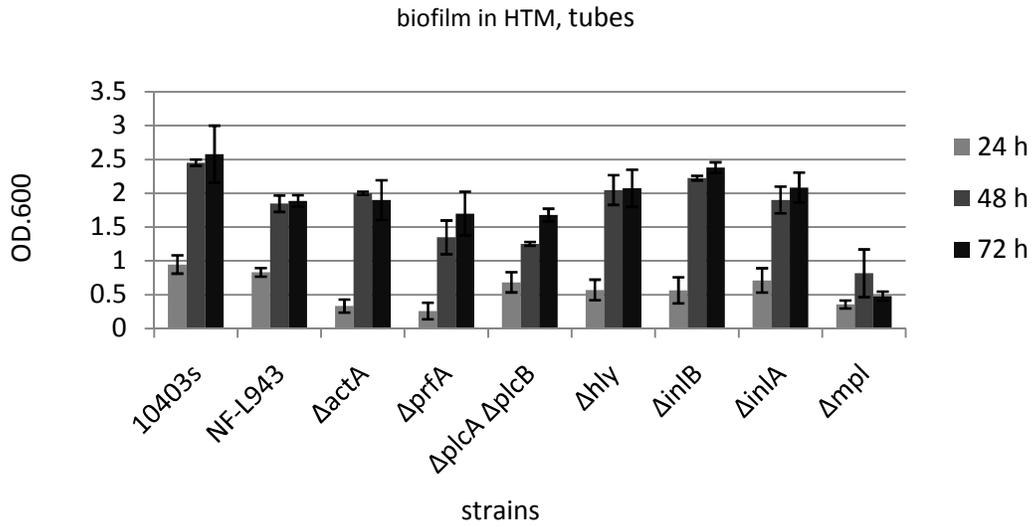Figure 4-9. Average biofilm formation of the PrfA mutant and virulence mutants from 3 parallel repeats in HTM medium in polystyrene tubes compared with the wild type 10403s strain.

CHAPTER FIVE

DISCUSSION

The adapted ability of biofilm formation can dramatically facilitate the bacterial transmission and infection. *L. monocytogenes* has been demonstrated to be capable of biofilm growth, which definitely increases its survival opportunity and chances to cause serious infections. Although several groups have identified a few functional molecules[52,57,73] that play critical roles in *L. monocytogenes* biofilm development, transcriptomic[125,126] and proteomic[127-129] studies on other species have also been carried out to generate comprehensive views of this process. It's a very complicated phenomenon affected by so many factors that currently a well-understood network is still lacking. In this study, a genome-wide screening for the functional factors involved in *L. monocytogenes* biofilm was performed through *mariner* transposon mutagenesis in combination with microtiter plate assays. The *mariner* transposon was initially constructed based on *Himar 1 mariner*, and modified specially for the transposition in low GC content Gram-positive microorganisms[114] with advantages including high transposition efficiency, good randomness, and low plasmid retention rate[114]. From 10,000 mutants screened, 14 were selected for an at least 50% reproducible biofilm defects comparing with the wild type. The growth rate of these 14 mutants was compared with the wild type, and none of them showed any growth defect, excluding the possibility of impaired growth of these mutants in our tests. The linkage test results suggested that

the transposon insertion in the bacterial chromosome is responsible for the observed biofilm defects.

The transposon insertion site in the 14 mutants were identified, and the genes involved were categorized into 5 different functional groups as shown in table 3-1, including transcriptional regulation, bacterial cell wall synthesis, flagella assembly, ATP formation and metabolism.

A big group of genes we've identified relates to cell wall components biosynthesis, especially lipoteichoic acid (LTA). LTA is a secondary wall polymer that consists of the cell wall of Gram positive bacteria with peptidoglycan, proteins, and capsular polysaccharides[130]. Normally LTA has a basic structure in which a poly-glycerolphosphate chain linked with the membrane glycolipid[131]. It is known that the glycerolphosphate in *L. monocytogenes* could be substituted by glycosyl residues or D-alanine esters[131]. LTA is a macroamphiphile molecule which by its electric charge properties exerts several functions in the Gram positive bacteria, including protection against environmental stress[132], regulation of cation concentration in the cell wall[133], and interaction with host cells[134], while D-alanylation of LTA directly relates to the electric charge properties of this polymer. LTA is an anionic polymer, and the D-alanine ester formation requires the anionic glycerolphosphate group and the cationic D-alanine group. The D-alanine ester content was suggested to determine the number of available anionic sites on LTA for binding with other cationic substances[133], such as autolysins, the enzymes catalyzing the hydrolysis of covalent bonds in the peptidoglycan of cell

wall[135]. So it seems like without functional D-alanine ester more autolysins would be able to bind to the LTA, and result in increased autolysis. This idea possibly explains the identification of the gene *lmo0106* in our study. *lmo0106* encodes the DltD protein for D-alanine esterification of LTA. It's reasonable that insertion mutation of DltD jeopardized the D-alanine ester substitution of LTA, which subsequently led to increased autolysins binding to the LTA in the mutant, increased cell lysis and reduced cell number in the biofilm community. A similar autolysis up-regulation was observed in *Lactobacillus rhamnosus* D-alanylation mutant[133].

Other genes in this group include *lmo1370* encoding a branched chain fatty acid kinase that transfers a phosphate group from the ATP to 2-methylpropanoate, *lmo2229* encoding a penicillin-binding protein which is involved in peptidoglycan biosynthesis, *lmo2553* encoding a hypothetical protein, *lmo2554* encoding a galactosyltransferase which is responsible for the glycolipid anchor production of LTA[131], and *lmo0644* encoding the LTA primase that mediates the transfer of initial glycerolphosphate to the glycolipid [131]. Huebner's group recently discovered that the glycolipids of *Enterococcus faecalis* is involved in biofilm formation[136]. The putative glucosyltransferase mutant exhibited significant alteration of membrane glycolipid profile and failed to accumulate in growing biofilm, although the initial adhesion was not affected[136]. LTA synthesis was proven to be required for *S.aureus* growth at 37°C[137], and the destruction of polyglycerolphosphate synthase resulted in aberrant cell division and separation[137]. In our study, it seems like the LTA is required for *L. monocytogenes* biofilm formation. In-frame deletion of *lmo2554* resulted in a around 90%

biofilm reduction while in-frame deletion of *lmo2553* led to a around 80% biofilm reduction at 37°C in HTM medium. In LB medium the biofilm reduction effect due to the absence of these two genes was not as significant as in HTM medium. To further support the role of cell wall components in *L. monocytogenes* biofilm, deletion mutants of the other identified genes should be tested for biofilm growth, and the respective genetic complementation should be performed as well to confirm their involvements.

Three transcriptional regulators were identified in our test. In the TM-1 transposon mutant, the transposon located at the 150 bp intergenic region between *lmo1262* and *lmo1263*. The transposon inserted between 32 bp and 33 bp upstream of *lmo1262*. It's possible that this insertion probably affected the promoter function and the subsequent transcription of both genes. *lmo1262* encodes a phage-related transcriptional regulator. Bioinformatic analysis of the protein sequence indicates that it is a phage λ repressor-like DNA binding protein. λ repressor DNA binding protein controls the expression of viral genes involved in lysogeny/lytic growth switch. This type of repressor is essential for maintaining the lysogeny cycle, and lytic growth is only induced when the host cell is threatened. It usually contains two domains connected by a linker: an N-terminal DNA-binding domain which also mediates the interaction with RNA polymerase, and a C-terminal dimerisation domain. The HTH motif of Lmo1262 locates at the N-terminal from 5th to 63rd amino acid. Analysis of *lmo1263* suggests that it also encodes a HTH-XRE transcriptional regulator, but is not phage-related. The HTH motif locates from 13th amino acid to 69th amino acid. *lmo0734* encodes a PurR transcriptional regulator, the N-terminal of which contains a HTH binding domain of Lac I family transcriptional

regulator. It was well proved that PurR and Lac I family transcriptional regulators have highly homologous secondary and tertiary structure[138]. This probably implies that the Lmo0734 regulator binds to DNA via its N-terminal domains to repress downstream gene transcription, and this repression can be retrieved through the interaction with a small effector ligand in a cleft of core N- and C-terminal intermediate region. The identification of the downstream genes regulated by these transcriptional regulators will reveal more valuable information about the underlying mechanisms of *L. monocytogenes* biofilm. Chromatin immune-precipitation (CHIP) based on the affinitive interaction between transcriptional regulator and its antibody could be a good method to pursue for this purpose.

Flagellum is a tail-like structure protruding from the cell body that generally mediates the bacterial extracellular movement. Flagellum biosynthesis plays an important role in *L. monocytogenes* extracellular motility, and several groups have reported different roles of flagellum in *L. monocytogenes* biofilm development. Though flagellum was generally considered as essential during the whole biofilm developmental process[52], Young's group did provide different evidences supporting the idea that flagellum was only required for the initial cell attachment, and absence of flagellum led to a final increased biofilm growth[53]. The two genes identified in our study actually function in different aspects of flagellum. *lmo0685* encodes a flagellum biosynthesis protein similar to MotA, which with MotB together forms the ion channels that couple flagellar rotation to the proton motive force across the membrane[139]. MotA mutation should destroy the ion channels to some extent and probably cut off the rotor power for

flagella rotations. Identification of this gene indicates that flagellar motility might be required for *L. monocytogenes* biofilm growth in our conditions. The other gene *lmo0707* encodes the flagellar hook-associated protein FliD. This protein contributes to the flagella functions by facilitating the polymerization of the flagellin monomers at the tip of growing flagellum filament. FliD forms a cap-like structure, which prevents the flagellin subunits from slipping out without polymerization at the end. Thus destroying the FliD was expected to jeopardize the formation of regular flagellum tail. It seems like the flagella tail-like structure is also involved in *L. monocytogenes* biofilm growth. It was well documented that flagellum biosynthesis is dramatically shut down when the bacterium senses the intracellular signal, such as 37°C which is the normal human body temperature. Initially in this study various biofilm growth conditions were tested for selection of optimal combination for biofilm growth, and 37°C reproducibly yielded better biofilm growth than 30°C. This probably suggests that a tiny amount of flagellum is enough for biofilm growth in our test condition.

lmo2205 which encodes a protein similar to phosphoglyceromutase 1 was identified in our screening. Phosphoglyceromutase catalyzes the inter-conversion of 2-phosphoglycerate and 3-phosphoglycerate, and it's generally involved in carbohydrate degradation and glycolysis. Another identified gene is *lmo0086*, which encodes the phosphoribosylformylglycinamidine synthetase I. This enzyme catalyze the production of ADP, phosphate, 2-(formamido)-N(1)-(5-phospho-D-ribosyl) acetamidine, and L-glutamate from a combination of substrates including ATP, N(2)-formyl-N(1)-(5-phospho-D-ribosyl) glycinamide, L-glutamine and H_2O. *lmo1083* encoding a dTDP-D-

glucose 4,6-dehydratase was identified in other project, and was found to involve in biofilm formation in LB medium, since absence of this gene led to reduced biofilm in LB medium, but not in HTM medium. It's possible that some ingredients from the LB complex medium specifically influence the sugar metabolism, and require the dTDP-D-glucose 4,6-dehydratase to assume a normal biofilm development. More information is needed to further characterize the role of these three genes in biofilm formation, considering that carbohydrate metabolism could possibly affect several aspects, such as bacterial physiology and matrix production, which could be relevant to normal biofilm growth.

Another group of genes identified in our screening includes *lmo2534*, *lmo2535* and *lmo2529*, all of which encode a subunit of ATP synthase. ATP is the primary energy supplier for most physiological and biochemical activities. Thus blocking ATP biosynthesis could interfere a lot of biological functions, including biofilm formation. It's noteworthy that the transposon mutants from this group did not show any growth defect. This might be due to the redundant functions of other subunits in the ATP synthase.

Interestingly in our screening, we haven't identified any increased biofilm mutant. Neither did we find any gene previously proven to mediate *L. monocytogenes* biofilm. Our bioinformatic study indicated that there are some homologues of *B. subtilis* proteins which have been shown to regulate biofilm, such as *lmo0168* that shares 60% identity with the AbrB repressor, or *lmo0806* that shares 35% identity with SinR repressor. No such homologue was turned up during our screening either. One possible reason for this

flaw is that among the 10,000 mutants we have screened several could be siblings, thus we actually need to screen more mutants to fully cover the whole genome of *L. monocytogenes*. The test conditions we adopted could be another possible reason. Biofilm formation is the type of microbial activity that closely relates with its surrounding environment. A minor variation in the test condition could lead to big differences in the results. The third possibility is that those essential genes which might also affect biofilm formation would not be found in our screening due to the limits of the method we used.

It was recently reported that besides mediating the transition from an extracellular free-living style to an intracellular pathogen, the master regulator of *L. monocytogenes* virulence, PrfA also contributes to normal biofilm growth[41]. Besides the genome-wide screening for functional factors in *L. monocytogenes* biofilm, we also tried to probe the role of PrfA in biofilm. Consistent with the reported result, deletion of PrfA led to a dramatic reduction in biofilm formation in minimal medium, while constitutive expression of PrfA had minimal effect. Surprisingly different from the reported, two virulence mutants, *plcA⁻plcB⁻* double deletion mutant and *mpl* mutant, showed obvious biofilm defects in our study. The biofilm reduction for *plcA⁻plcB⁻* double mutant was similar to that of the PrfA mutant at 48 h and 72 h, while the reduction of *mpl* mutant was even more significant than that of the PrfA and double mutant. It would be interesting to investigate whether the contribution of PrfA in biofilm is related with its regulatory effect on the virulence genes, or other unidentified factors. *mpl* encodes the zinc metalloprotease which is responsible for the maturation of the broad-range phospholipase

C (PC-PLC) encoded by *plcB*. It contributes to *L. monocytogenes* virulence by cleaving the N-terminal signal peptide of PC-PLC. This cleavage activates the PC-PLC enzyme activity that is important for bacterial escape from the vacuole. If Mpl contributes to biofilm growth only by function of PC-PLC, a similar defect of *mpl⁻* mutant and *plcB⁻* mutant would be expected. However the variation between our *mpl⁻* mutant and *plcA⁻ plcB⁻* double mutant seems to indicate that Mpl might have another downstream target, and this target also plays a role in biofilm growth. It will be more valid to compare the biofilm formation of *plcB⁻* mutant with *mpl⁻* mutant. Also it will be interesting to look at whether the enzyme activity of PC-PLC is required for biofilm or not.

In conclusion, we identified 16 genes that are possibly involved in *L. monocytogenes* biofilm formation by transposon mutagenesis in combination with microtiter plate assay. These genes play a role in bacterial transcriptional regulation, cell wall synthesis, flagella assembly, ATP formation or metabolism. The detailed functions of these genes in biofilm development await further study through the non-polar mutants and the signal pathways involved. This study will generate several valuable information about the molecular basis of *L. monocytogenes* biofilm formation, which will eventually help with the identification and development of drug targets for biofilm eradication.

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