Genome-wide screening of genes required for *Listeria monocytogenes* biofilm formation

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***Listeria monocytogenes* is a ubiquitous Gram-positive food-borne pathogen, which can cause serious infections in immunocompromised individuals due to the intake of *L. monocytogenes* contaminated food. In addition to planktonic growth, this pathogen can also grow as biofilms. Biofilm bacteria are highly resistant to sanitizers and are thus difficult to eradicate, posing a big challenge to food processing industries and food service departments. Here, we report the results from a systematic genome-wide study of genes involved in biofilm formation. Using transposon mutagenesis in combination with microtiter plate assays, we surveyed 10,000 transposon mutants. 17 mutants were isolated that showed more than 50% decrease in biofilm formation. The insertions were localized within 13 genes (11 operons) and two intergenic regions, which affect various cellular pathways such as wall and lipoteichoic acid biosynthesis and modification, cell wall turnover, flagella synthesis, ATP synthesis, and transcription regulation. Of these genes or regions, only the two flagella-related genes were previously known to be crucial for *L. monocytogenes* biofilm formation. All other genes are newly identified from our library screen. Further investigations on these genes will provide a better understanding of the molecular basis of *L. monocytogenes* biofilm formation, which will undoubtedly reveal important insights on effective measures to prevent and control contaminations in the food industry.**

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Introduction

Biofilms are generally considered as aggregates of microbial cells that attach to either biotic or abiotic surfaces and grow as matrix-encased communities. The cells found in biofilms are phenotypically different from their planktonic counterparts in several ways: they express different sets of genes and communicate intercellularly through signal molecules, they produce extracellular polysaccharides (EPS) upon attachment to surfaces, and they are highly resistant to sanitizers and antibiotics due to the protection provided by the EPS matrix. Naturally occurring biofilms can grow on processing equipment and potentially contaminate all of the downstream products, which may cost billions of dollars each year from food contamination, energy loss, and machinery corrosion. Moreover, biofilms have been found to be involved in a wide variety of microbial infections in the human body, such as urinary tract infections, catheter infections, formation of dental plaque, gingivitis, and coating contact lenses. Both Gram-negative and Gram-positive bacteria, including the food-borne bacterial pathogen *Listeria monocytogenes*, have been shown to be capable of biofilm growth on the surfaces of various materials.

L. monocytogenes is a Gram-positive bacterium, ubiquitously distributed in the environment. As the etiologic agent of listeriosis, it can cause serious infections in newborns, elders, pregnant women, and immunocompromised individuals [1, 2]. According to recent statistics from the Center of Disease Control, about 1,600 cases of listeriosis occur annually in the US, with more than 1,400 hospitalizations and 250 deaths [3].

The mortality rate is much greater than that caused by *E. coli* O157:H7. The outbreaks of listeriosis are usually associated with food contaminations [4]. Dairy products, raw meat and sea food are the common niches for *L. monocytogenes*, and various Listeria serotypes have been isolated from meat and dairy processing plants. In the US, a recent listeriosis outbreak due to contaminated cantaloupes has quickly spread from Colorado to 28 states within three months and has already caused 29 deaths and one case of miscarriage [5]. *L. monocytogenes* is notorious for its ability to survive under a diverse array of deleterious environmental conditions such as freezing temperature, acidic pH, and high salinity [6]. In addition, it was shown that *L. monocytogenes* can attach quickly to the surfaces of stainless steel and buna-n rubber (two materials commonly used in food processing equipment) and form biofilms [7].

Extensive studies on biofilm development in various bacteria have led to a general five-stage developmental process: initial surface contact, irreversible attachment, matrix production and architecture development, maturation, and cell dispersion from the biofilm [8]. The mechanisms and mediators of the cell attachment have been a focus of biofilm research for a long time. To date, flagella are considered to play an important role on seed cell attachment in several bacteria such as *E. coli* [9] and *Pseudomonas aeruginosa* [10]. In *L. monocytogenes*, several genes related to regulation of flagella synthesis (*degU*), flagella structure (*flaA* and *flgL*) and motility (*motB*) have been linked to biofilm formation [11-14]. Besides flagella, the bacterial quorum sensing (QS) systems also play a role in the early stages of biofilm formation. In *L. monocytogenes*, the peptide-mediated QS system (Agr) and the autoinducer 2 (AI-2) LuxS system are both involved. In the Agr system, disruption of the signal peptide AgrD or the response regulator AgrA resulted in decreased biofilms within the first 24 hours, but did not affect the maturation stage [15]. On the other hand, LuxS seems to play a negative role since deletion of *luxS* led to a denser biofilm [16, 17]. However, the real role of LuxS in biofilm formation was suggested to be irrelevant to QS as addition of AI-2 molecules could not restore the normal biofilm level. But addition of the AI-2 precursor, S-ribosyl homocysteine (SRH), stimulated early cell attachment. Thus, LuxS plays an indirect role in biofilm repression by converting SRH into AI-2 [16]. The extracellular matrix of biofilms is comprised of proteins, EPS, lipids, and nucleic acids [8]. It is reasoned that factors with the ability to regulate the components of biofilm matrix will control or eliminate biofilm growth. In *L. monocytogenes*, extracellular proteins [18] and DNAs [19] have been characterized as vital for the initial adhesion and matrix formation. Other factors that only affect the late stages of biofilm development have recently been identified in *L. monocytogenes*. These include the major virulence regulator PrfA [20, 21], the guanosine pentaphosphate synthetase RelA [22], the 6-oxopurine phosphoribosyltransferase Hpt [22], and the SOS response related proteins YneA and RecA [23].

Despite the dedication of many studies on biofilm developmental process in various bacterial species, the molecular mechanisms of *L. monocytogenes* biofilm formation are still not well understood. To address this question systematically, we employed genome-wide transposon mutagenesis in combination with crystal violet based microtiter plate assays to identify *L. monocytogenes* genes that are involved in biofilm development. This screen surveyed 10,000 transposon mutants, which covered three times the *L. monocytogenes* genome. 17 insertion mutants were isolated that showed a more than 50% decrease in biofilm formation. The insertions were localized within 13 genes (11 operons) and two intergenic regions, which affect various cellular pathways such as wall and lipoteichoic acid biosynthesis and modification, cell wall turnover, flagella synthesis, ATP synthesis, *etc*. Of these genes or regions, only the two flagella-related genes were previously known to be

crucial for *L. monocytogenes* biofilm formation. All other genes were newly identified from our library screen. We are the first to report the results from a systematic genome-wide survey of genes involved in biofilm formation. Our findings provide an important entry point for further characterizations, which will certainly reveal important insights into the molecular control of *L. monocytogenes* biofilm formation.

Materials and methods

Strains and growth conditions.

The *L. monocytogenes* wild type strain 10403S (serotype 1/2a) [24] and the *flaA* in-frame deletion strain HEL-304 [25] were obtained from Hélène Marquis, Cornell University, Ithaca, NY, USA. Planktonic *L. monocytogenes* was cultured in Brain Heart Infusion medium (BHI) (Bacto, USA) at 37°C. *L. monocytogenes* biofilms were grown statically in HTM medium [26] or other media as indicated at 37°C. In *L. monocytogenes*, 200 μg/ml of streptomycin was used to select for $Strep^R$, and 5 μg/ml of erythromycin was used to select for Em^R.

Static biofilm growth and quantification.

Overnight *L. monocytogenes* culture was 1:100 diluted into the HTM medium or other tested media in 5ml polystyrene round-bottom tubes (BD Falcon). The cultures were incubated statically at 25°C, 30°C, or 37°C for 24h, 48h and 72h. To obtain quantitative results of biofilm formation, the supernatant of bacterial cultures was discarded from each tube and the tube was washed three times with 5ml of phosphate buffered saline solution (PBS). The sessile bacterial cells were stained with 1% of crystal violet for 30 minutes, followed by distilled water wash for 5 times. To destain, 1ml of a mixture of 80% ethanol and 20% acetone was added to each tube for at least 1h to solubilize the crystal violet that had stained the biofilm cells. The quantity of biofilm formed in each tube was indicated by the absorbance at 595nm (Bio Rad - SmartSpec Plus).

Construction of *mariner*-insertion libraries and screening of biofilm mutants.

Random transposon-insertion libraries of *L. monocytogenes* strains were constructed using the *mariner*-based transposon system as described previously [27]. Approximately 10,000 insertion mutants from 20 libraries were randomly picked and cultured individually in 200 μl of BHI medium with 200 μg/ml of streptomycin and 5 μg/ml of erythromycin in 96-well polystyrene microtiter plates at 37°C overnight without shaking. 5 μl of the overnight culture from each well was transferred into 200 μl of HTM medium in a new 96-well polystyrene plate for biofilm development. Listeria biofilms were allowed to grow at 37°C for 48 hours before quantitative measurement. A similar crystal violet staining method as described for the tube assay was applied to the microtiter plates. The absorbance was measured using the MULTISCAN EX plate reader (Thermo, PA, USA). The wild type strain 10403S and the *flaA* in-frame deletion strain were included in each 96-well plate as the positive and negative controls, respectively. Mutants that resulted in at least a 50% decrease in biofilm formation compared to that of the wild type were selected for further characterization.

Characterization of *mariner*-insertion mutants.

Each mutant strain was infected with phage U153 [28] to generate a phage library. The wild type 10403S strain was then transduced with the phage library with selection for Em^R. The resulting transductants were tested for biofilm formation. Only those mutants whose phenotypes showed decreased biofilm formation were linked to the *mariner* Em^R marker and were picked for further analysis. To determine the loci in which the transposons were inserted, we used arbitrary-primed nested-PCR [29] followed by DNA sequencing as described previously [27]. The sequence of chromosomal DNA flanking the transposon was compared with the *L. monocytogenes* EGD-e genome using the BLAST program [30] available on the ListiList website at http://genolist.pasteur.fr/ListiList/.

Results

Construction of *mariner*-based transposon insertion libraries in *L. monocytogenes.*

A *mariner-based* transposon system was developed and has been proven to be an ideal genetic tool to study *L. monocytogenes.* Compared to the routinely used Tn917 mutagenesis system, the *mariner* system has 20-fold higher transposition efficiency and is very random [27]. To support our systematic survey in *L. monocytogenes*, we applied the *mariner* system and constructed twenty independent transposon insertion libraries. Approximately 10,000 mutants, i.e., ~500 colonies per library, were collected for this study. As the *L. monocytogenes* genome encodes around 3,000 genes, our library has roughly 3-fold coverage of the genome. Frozen stocks of all the mutants were prepared and maintained at -80^{o}C, which provided a large number of mutants not only useful for this study but also for any future genetic screens in *L. monocytogenes*.

HTM medium promotes *L. monocytogenes* 10403S biofilm growth.

To ensure the efficiency and accuracy of our screens, we optimized the growth condition for *L. monocytogenes* biofilm formation, which stimulates robust biofilm growth. We compared different media and temperatures on *L. monocytogenes* biofilm development by growing the wild type strain 10403S and the *ΔflaA* strain (as negative control) in 5ml polystyrene round-bottom culture tubes. As shown in Figure 1, we found that the nutrient-limited medium HTM allowed for the highest biofilm growth of the wild type strain as compared to the LB or BHI medium regardless of the temperature, and 37°C was better than 30°C and 25^{o}C for 10403S strain biofilm growth. The results were reproducible when conducted in the 96-well polystyrene microtiter plates. Taken together, 37°C and HTM were selected as standard conditions for the subsequent assays.

Isolation and identification of mutants with decreased biofilm formation.

Using the high-throughput microtiter plate assay coupled with crystal violet staining, we tested roughly 10,000 *mariner* insertion mutants derived from the wild-type 10403S strain for their abilities to form biofilms. In general, individual mutants were inoculated in HTM medium and grown statically at 37^{o}C for 48 hours. Any mutant showing at least a 50% reduction of biofilm formation as compared to the wild type was scored positive and picked for further analysis. Initially, a total of 121 mutants were isolated, all showing weaker biofilms.

The 121 mutants were first tested for growth in the HTM medium with and without shaking to rule out insertions that led to growth defects. Mutants with regular growth rates were checked again in polystyrene culture tubes for their abilities to form biofilms in a 3-day time course. Mutants that showed a more than 50% reduction in biofilm formation were subsequently subjected to genetic linkage tests by using the phage U153 transduction system [28]. Only those mutants whose phenotypes were linked to the *mariner* Em^{R} marker were selected for further characterization. In brief, we have isolated 17 mutants that were defective in biofilm formation. All these mutants showed more than a 50% reduction of biofilm formation from day 1 (24hr) to day 3 (72hr) (Figure 2).

Transposon flanking DNA was PCR amplified and sequenced for each mutant. A total of 13 genes (11 operons) and two intergenic regions were identified from the 17 mutants (Table 1). These mutants were clustered into four functional classes: (i) cell envelope biosynthesis and homeostasis, (ii) flagella and motility, (iii) energy generation and intermediary metabolism, and (iv) transcription regulation.

Cell envelope biosynthesis and homeostasis.

We identified four genes from five *mariner*

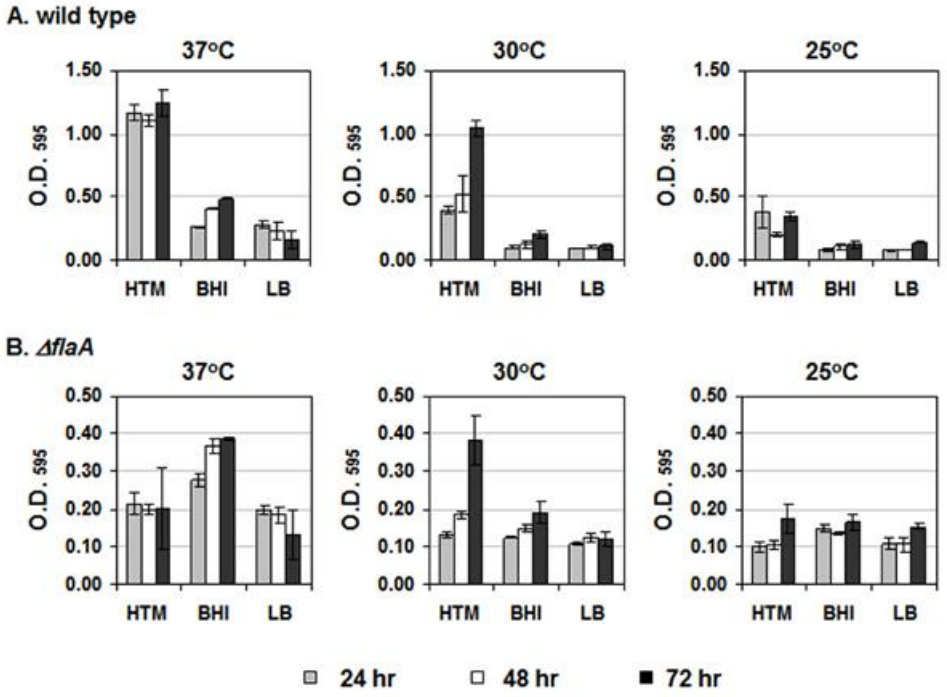

Figure 1. Biofilm formation in polystyrene tubes by *L. monocytogenes* wild type 10403S (A) and the *ΔflaA* (B) in 3 different media (HTM, BHI, LB) and at 37°C, 30°C, and 25°C. Biofilms formed by the two strains were measured after 24, 48, and 72 hours of static incubation. The result is the average and standard deviation from three individual measurements. Three parallel samples were tested per measurement.

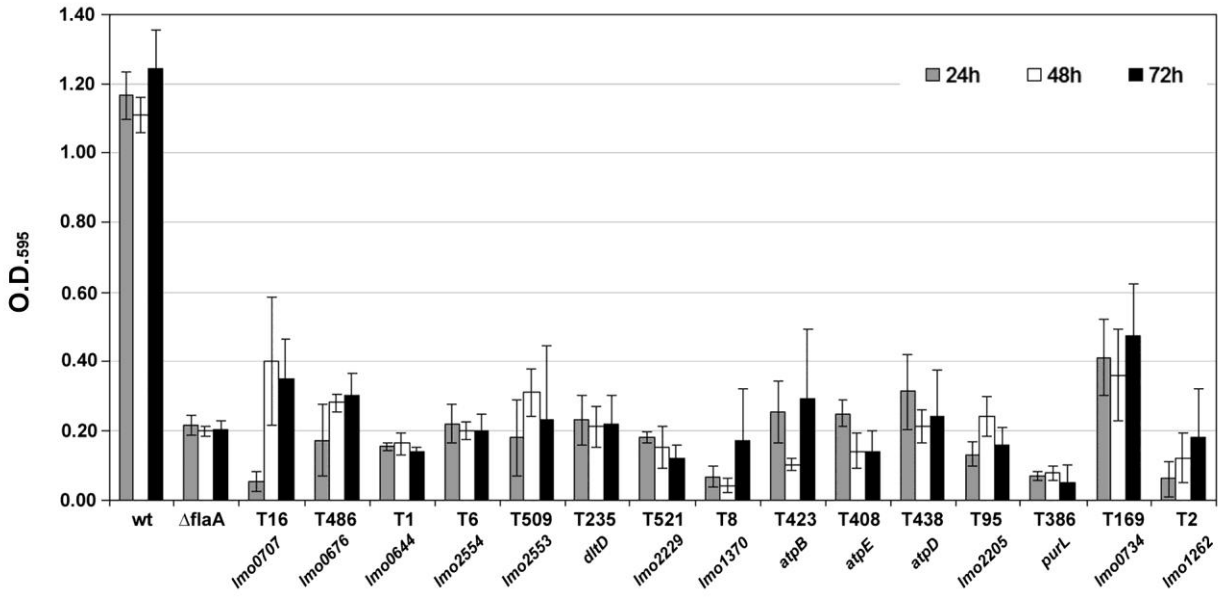

Figure 2. Three-day biofilm formation in polystyrene tubes by the *mariner* insertion mutants as compared to the *L. monocytogenes* wild type 10403S and the negative control *ΔflaA* in HTM at 37°C. The result is the average and standard deviation from three individual measurements. Three parallel samples were tested per measurement.

insertion mutants (T1, T6, T92, T235, and T509) whose functions all seem to be related to cell wall teichoic acid (WTA) and lipoteichoic acid (LTA) synthesis and modification. WTA and LTA polymers are common components found on the cell surface of Gram-positive bacteria. In *L. monocytogenes*, the basic structure of LTA is a chain of polyglycerolphosphate linked to the glycolipid of the membrane. Genes *lmo2553* and *lmo2554* are in the same operon. In addition, there is another gene, *lmo2555*, in this operon that is located upstream of *lmo2554* and *lmo2553*. A recent study suggested that both Lmo2555 (also named LafA) and Lmo2554 (also named LafB) are cytoplasmic glycosyltransferases that are involved in the synthesis of the glycolipid Gal(α1-2)Glc(α1-3)-diacylglycerol (Gal-Glc-DAG), the linker which connects LTA to the bacterial membrane. Lmo2553 is a membrane protein whose function is not clearly defined, but it is proposed to function downstream of LafA and LafB in the glycolipid biosynthesis pathway [31]. Gene *lmo0644* also encodes a membrane protein, whose function was suggested as the LTA primase (LtaP) which transfers the initial glycerolphosphate onto the glycolipid linker, followed by another transmembrane protein LtaS (encoded by *lmo0927*) that transfers additional glycerolphosphate molecules onto the growing LTA chain [31]. In most Gram-positive bacteria, the mature WTAs and LTAs are usually modified with D-alanine residues, and this modification is accomplished by four proteins encoded in the *dltABCD* operon. DltC is the D-alanyl carrier protein. DltA is the activating enzyme that links D-alanine to DltC. DltB and DltD are responsible for the transport and the D-alanine esterification reaction. We identified a *mariner* insertion in *dltD*, the fourth gene in the operon. Therefore, we have characterized four genes (*lmo2553*, *lmo2554*, *lmo0644* and *dltD*) related to teichoic acid biosynthesis and modification. Transposon insertions in these genes resulted in dramatic decreases (from 72% to 85%) of *L. monocytogenes* biofilm (Table 1), yet none had any effect on bacterial growth (data not shown). Though WTAs and LTAs have been reported to contribute to biofilm development in several bacteria, we are the first to elucidate this in *L. monocytogenes*.

Mutant T521 possesses a *mariner* insertion in *lmo2229*, which encodes a putative penicillin-binding protein (PBP). PBPs are one of the major components of bacterial cell wall that are usually involved in the final stages of the peptidoglycan synthesis. Previously, Zawadzka-Skomiał *et al*. mutated gene *lmo2229* but they did not detect any obvious phenotypes such as growth deficiency, changes in peptidoglycan composition, cell morphology, or sensitivity to β-lactam antibiotics. However, the synthesis and content of peptidoglycan in *Δlmo2229* were found to be slightly lower than those in the parent strain [32]. In contrast, an insertion mutation in *lmo2229* generated by Hill's group resulted in not only sensitivity to β-lactam antibiotics, but also altered morphology and attenuation in virulence [33]. The *lmo2229* mutant obtained from our screen showed an 86% reduction of biofilm, indicating that PBP-modulated cell wall turnover is crucial for *L. monocytogenes* biofilm formation. Other PBPs have also been studied by several labs [32, 34-39], however, there are not any reports on PBPs' role in Listeria biofilm development.

Mutants T8 and T489 individually disrupted gene *lmo1370*, which encodes a branched-chain fatty-acid (BCFA) kinase. Lmo1370 and its downstream genes are involved in BCFA biosynthesis. Previous studies have shown that BCFAs represent the majority of membrane fatty acids in *L. monocytogenes* and contribute to overall membrane fluidity and resistance against environmental stresses [40-42]. In this study, we found that insertion in *lmo1370* almost completely prevented sessile growth, indicating BCFAs' significant role in biofilm formation. Interestingly, in the initial screen we also isolated mutations in *lmo1372* and *lmo1374* (encoding the branched-chain alpha-keto acid dehydrogenase E1 and E2 subunits). However, mutations in these two genes (not in

Table 1. Summary of *mariner* insertion mutants that are defective in biofilm development.

Strain	Gene	*mariner* insertion site [a]	Gene function or feature [b]	% decrease of biofilm [c]
(i) Cell envelope biosynthesis and homeostasis				
T1	*lmo0644*	840	Unknown	85
T6, T92	*lmo2554* [d]	917	Similar to galactosyltransferase	82
T509	*lmo2553* [d]	121	Unknown	72
T235	*dltD*	840	D-alanine esterification of lipoteichoic and wall teichoic acid	81
T521	*lmo2229*	178	Similar to penicillin- binding protein	86
T8,T489	*lmo1370*	261	Similar to branched chain fatty acid kinase	96
(ii) Flagella and motility				
T486	*lmo0676*	319	Similar to flagellar biosynthetic protein FliP	75
T16	*lmo0707*	469	Similar to flagellar hook-associated protein 2 FliD	63
(iii) Energy generation and intermediary metabolism				
T423	*atpB* [e]	576	Highly similar to H^+-transporting ATP synthase chain a	91
T408	*atpE* [e]	-37	Highly similar to H^+-transporting ATP synthase chain c	87
T438	*atpD* [e]	1034	Highly similar to H^+-transporting ATP synthase chain beta	83
T95	*lmo2205*	389	Similar to phosphoglyceromutase 1	78
T386	*purL*	506	Phosphoribosylformylglycinamidine synthetase II	93
(iv) Transcription regulation				
T169	*lmo0734*	551	Similar to transcriptional regulator (LacI family)	68
T2	*lmo1262*	-32	Similar to transcriptional regulator (phage-related)	89

[a] Relative to the start codon of the gene.

[b] Gene functions or features were based on the information from the ListiList database (http://genolist.pasteur.fr/ListiList/).

[c] % decrease of biofilm was calculated as such: (OD_{595}WT - OD_{595}Mutant) ÷ OD_{595}WT × 100%. Based on the 48-hour biofilms.

[d] The genes belong to the same operon (*lmo2555-lmo2554-lmo2553*).

[e] The genes belong to the same operon (*atpIBEFHAGDC*).

lmo1370) caused a marked growth defect not only in HTM medium but also in the rich medium BHI.

Flagella and motility.

Two *mariner* insertion mutants (T16, T486) apparently affected flagella biosynthesis. *lmo0707* encodes a protein similar to the flagellar hook-associated protein FliD, while *lmo0676* encodes a protein similar to the flagellar biosynthetic protein FliP. Both mutants showed a more than 60% decrease in biofilm formation, which is comparable to that of the negative control *ΔflaA* strain (Figure 2). Flagella are tail-like structures protruding from the cell body that confer motility to bacteria. Previous studies from Kolter's lab suggested that flagellum-mediated motility is critical for not only the initial surface attachment but also the subsequent biofilm development [12]. Our results were consistent with Kolter's and support the view that flagellum-mediated motility is essential for *L. monocytogenes* biofilm formation under static conditions.

Energy generation and intermediary metabolism.

Three mutants disrupted in the *atpB*, *atpE*, and *atpD* genes respectively showed a great reduction of biofilm production. For *atpE*, the insertion is 37bp upstream of the start codon. They are the 2nd, 3rd, and 8th genes of the *atpIBEFHAGDC* operon. The operon encodes nine proteins that constitute the large membrane complex, known as the ATP synthase. ATP is the primary energy supplier for most physiological and biochemical activities in the cell. Hence, disturbance of ATP biosynthesis will impair numerous biological processes. And it is logical to reason that any mutant in these genes will be either weak or lethal. However, none of the mutants showed any growth defect. This might be due to the existence of another ATP synthase operon (*lmo0088-lmo0093*) in the *L. monocytogenes* genome. From the Listeria database, we found that both *atpE* and *lmo0088* encode chain c, the major subunit of the ATP synthase F0 domain (the entry channel for protons). *atpD* and *lmo0092* both encode chain β, the key component of the catalytic F1 domain. Disruption of one copy of these genes may still support growth of planktonic cells, but it is detrimental to sessile cells. It is possible that one operon is responsible for planktonic growth while the other is for sessile. It will be interesting to construct mutations in the *lmo0088-lmo0093* operon and monitor the effects on biofilm production. Remarkably, a recent study by Xu *et al.* demonstrated that chemical inhibition of ATP synthesis could promote detachment of different-age biofilms from membrane surfaces [43], suggesting energy generation is crucial for biofilm development and maintenance.

Mutant T95 contains a *mariner* insertion in gene *lmo2205*. Lmo2205 is similar to phospho-glyceromutase 1, an enzyme in the glycolysis pathway. Mutant T386 has an insertion in *purL*, the gene for phosphoribosylformylglycinami-dine synthetase II. *purL* is located inside of the large *pur* operon, which is responsible for *de novo* purine nucleotide biosynthesis. The mutant showed a 93% reduction of biofilm yet no growth defect. As far as we know, these genes and pathways have not been reported to participate in bacterial biofilm formation. Yet an earlier proteomic study may support our notion. Protein patterns of *L. monocytogenes* grown in biofilm and in planktonic mode were compared. Expression of two key enzymes involved in global carbon metabolism (PdhD and Pfk) were up-regulated by biofilm growth, indicating that *L. monocytogenes* intermediary metabolism is affected by biofilm development [44].

Transcription regulation.

Two putative transcription regulators were identified from our screen. Mutant T169 contains a *mariner* insertion inside of gene *lmo0734*. This gene encodes a LacI family regulator. Mutant T2 has a *mariner* insertion within the intergenic region between *lmo1262* and *lmo1263.* The two genes are 151bp apart and transcribed in divergent directions (Fig. 3).

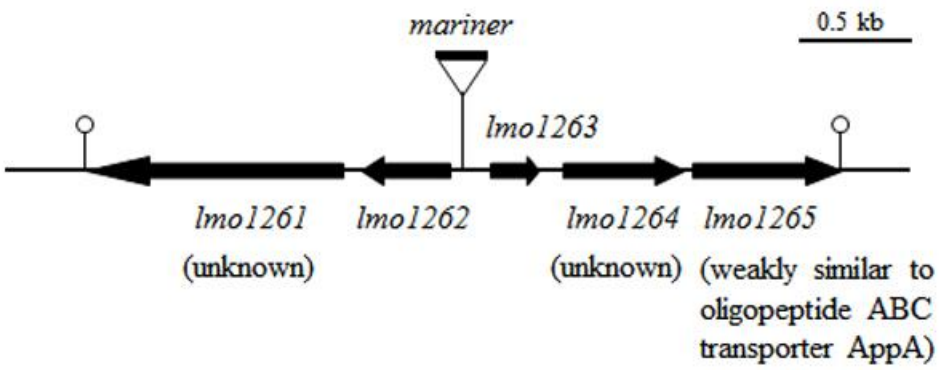

Figure 3. Diagram of the regions flanking gene *lmo1262*. The *mariner* insertion in mutant T2 is located in the intergenic region between *lmo1262* and *lmo1263*. Both genes encode putative phage-related transcription regulators. The proposed functions of other genes are listed. The lollipops stand for transcription terminators.

Insertion in this region could affect expression of both genes. Notably, sequence analysis indicates that both genes encode phage-related transcriptional regulators.

Validation of the mutants.

Transposon insertion inside of a gene will usually inactive that particular gene, but will also affect expression of the downstream genes of the same operon due to a polar effect. We checked the chromosome localizations of all the candidate genes. In most cases (such as *lmo2553*, *lmo2554*, *dltD*, *lmo1370*, *lmo0676*, *lmo0707*, *purL*, and *atpBED*), the gene disrupted by *mariner* insertion belonged to an operon that consists of genes of the same pathway. Thus, if there was a polar effect on downstream genes, it would only affect the same pathway. Therefore, it is reasonable to link the physiological function of this pathway (the entire operon) to biofilm development. Insertion in genes *lmo0644*, *lmo2229*, *lmo2205*, and *lmo0734*, will not generate any polar effects because these genes don't have any downstream genes. However, we cannot totally rule out the possible effects on upstream gene expression by transposon insertion. One insertion is in the intergenic region between *lmo1262* and *lmo1263*, which could affect transcription of both genes. Hence, construction of in-frame deletions and complementation tests are required for further investigation on these candidates.

Discussion

Biofilm formation has been proposed as an integral phase of the bacterial life cycle which is usually adopted when the bacterial cells confront unfavorable living conditions. Compared to its planktonic counterparts, biofilm bacteria have distinct patterns of gene expression and thus are physiologically dissimilar. To date, only a limited number of genes have been recognized in *L. monocytogenes* that play critical roles in biofilm development [45] and the molecular basis is still not very clear. In the present study, we applied a genome-wide screen in *L. monocytogenes* by using the recently developed *mariner* transposition system in combination with the high-throughput microtiter plate crystal violet straining assays to identify factors important for biofilm development. The *mariner* transposition system is an ideal tool for the genetic study of low GC content Gram-positive bacteria. It provides high transposition efficiency, good randomness, and low plasmid retention rate [27]. We screened 10,000 *mariner* insertion mutants, and 17 of them displayed a dramatic decline in biofilm production. The insertion sites were localized within 13 genes (11 operons) and two intergenic regions. In addition to the two flagella-related genes that are known to be important for biofilm formation, all the other genes are newly identified in this work.

The cell envelope of Gram-positive bacteria is an important structural compartment which consists of peptidoglycan, proteins, lipids, and capsular polysaccharides as well as secondary wall polymers such as WTA and LTA [46]. Alterations in the structure or composition of the envelope components will lead to a range of physiological changes such as cell morphology, surface attachment, autolysis, stress resistance, protein transport, and virulence. Thus, the properties of the cell envelope can directly influence bacteria-environment or bacteria-host interactions.

The penicillin-binding proteins (PBPs) participate in the final steps of peptidoglycan biosynthesis, which is the major cell wall component. Disruption of the PBPs will lead to abnormal cell shape, lesions, and even cell lysis. The sequenced *L. monocytogenes* EGD-e strain encodes 7 PBPs in its genome. The *lmo2229* mutant isolated from our screen showed an 86% reduction in biofilm formation yet no defects in planktonic growth rate, suggesting that these PBPs may have some redundant functions in peptidoglycan synthesis in planktonic cells. However, their expression and functions in sessile cells may be quite diverse. The roles of PBPs and peptidoglycan turnover in biofilm formation have been revealed in some other bacteria. In *Streptococcus gordonii*, mutation in genes encoding PBP B2 and PBP 5 caused 91% and 56% reduction of biofilm formation, respectively [47]. In the same study, two genes (*glmM* and *bacA*) involved in the early stages of peptidoglycan biosynthesis were also identified to be important for *S. gordonii* biofilm formation [47]. In *Lactococcus lactis*, peptidoglycan breaks were positively linked to cell adhesion and biofilm formation [48]. In *E. coli*, a Gram-negative bacterium, single deletions of PBPs 4, 5, and 7 led to a varying degrees of decrease (10-30%) in biofilm formation in the minimal medium, while a combined triple deletion resulted in almost 50% reduction of biofilm, indicating redundant functions of the three PBPs in *E. coli* [49]. These studies as well as our finding support the idea that PBPs and peptidoglycan turnover contribute to biofilm formation.

The pivotal role of WTA and LTA in biofilm formation has been addressed in several bacteria. Disruption of genes required for WTA and LTA synthesis [50-54] as well as their modifications [55, 56] caused reduced surface attachment and biofilm production. Moreover, the teichoic acid polymers have been isolated from *S. epidermidis* biofilm [57]. In this study, we identified four genes related to WTA and LTA synthesis and modification. Genes *lmo2553*, *lmo2254*, and *lmo0644* are involved in LTA synthesis [31], and *dltD* is required for further modification with D-alanine residues. This is the first report to elucidate the role of teichoic acids in *L. monocytogenes* biofilms.

Other factors identified from our screens that are essential for *L. monocytogenes* biofilm development include three subunits of the ATP synthase (*atpB*, *E*, *D*), enzymes for BCFA synthesis (*lmo1370*), sugar metabolism (*lmo2205*), and purine biosynthesis (*purL*), as well as two transcription regulators (*lmo0734* and *lmo1262*). It is worth noting that the *mariner* insertion site in mutant T2 is in the intergenic region, which is 32bp upstream of *lmo1262* and 118bp upstream of *lmo1263* (Fig. 3). Thus, transcription of both genes could be affected by the insertion. Interestingly, both genes encode phage-related transcriptional regulators. Their functions and gene arrangement on the chromosome are reminiscent of the CI/Cro proteins of the λ phage, which control the switch between lysogenic and lytic life cycles. Recently, a survey with a number of *L. monocytogenes* isolates suggested a possible connection between the existence of a prophage and the capacity for biofilm formation [58]. The wild type 10403S strain used in our study also harbors the prophage. It will be exciting to check whether or not these two transcription regulators are related to the prophage gene expression and to study their roles in biofilms.

The *L. monocytogenes* genome contains approximately 3,000 genes. In this study, we screened 10,000 colonies, which is 3-fold coverage of the entire genome. According to a previous study on mariner-based mutagenesis in *S. aureus*, at least 20,000 random insertion mutants are required to achieve saturating mutagenesis [59]. Obviously, our screen did not cover the entire genome. This partially explains why we didn't get other known biofilm-related genes except the two flagella genes. On the other hand, we used relatively stringent (> 50% of biofilm reduction) criteria in the screen. Thus, a number of mutants with mild reduction (20-50% reduction) would be missed. In addition, mutants that only affect the initial attachment step but can later catch up with the maturation process would not be isolated from our screen. We also did not isolate any mutants with stronger biofilms, probably because the screening method we used is not sensitive enough.

In conclusion, using the new *mariner*-based transposition system, we successfully identified 17 biofilm-defective mutants which are localized within 13 genes and two intergenic regions and are linked to various cellular pathways. The *Tn917*-based transposon libraries have been screened by several labs for *L. monocytogenes* biofilm-related genes, but to our knowledge, this is the first complete report from a systematic genome-wide survey. Our findings provide an important entry point for further characterizations, which will hopefully reveal important insights into the molecular control of *L. monocytogenes* biofilm formation.

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