

A Putative ABC Transporter Is Involved in Negative Regulation of Biofilm Formation by *Listeria monocytogenes*[∇]

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Listeria monocytogenes may persist for long periods in food processing environments. In some instances, this may be due to aggregation or biofilm formation. To investigate the mechanism controlling biofilm formation in the food-borne pathogen *L. monocytogenes*, we characterized LM-49, a mutant with enhanced ability of biofilm formation generated via transposon Tn917 mutagenesis of *L. monocytogenes* 4b G. In this mutant, a Tn917 insertion has disrupted the coding region of the gene encoding a putative ATP-binding cassette (ABC) transporter permease identical to Lmof2365_1771 (a putative ABC transporter permease) presented in the sequenced strain *L. monocytogenes* strain 4b F2365. This disrupted gene, denoted lm.G_1771, encoded a protein with 10 transmembrane helices. The revertant, LM-49RE, was obtained by replacing lm.G_1771::Tn917 with lm.G_1771 via homologous recombination. We found that LM-49RE formed the same amount of biofilm biomass as the wild-type strain. Furthermore, transcription of the downstream lm.G_1770 gene was not influenced by the upstream Tn917 insertion, and the presence of Tn917 has no effect on biofilm formation. These results suggest that lm.G_1771 was solely responsible for the negative regulation of biofilm formation by *L. monocytogenes* 4b G. The immediate gene upstream of lm.G_1771 encoded an ATP-binding protein. Bioinformatics analysis suggested that these two genes were organized into an operon and that their proteins formed an export ABC transporter. Here, we report the characterization of the mutant and identification of a novel ABC transporter that functions in negative regulation of biofilm formation in *L. monocytogenes*.

The gram-positive bacterium *Listeria monocytogenes* is responsible for listeriosis outbreaks and causes infections in fetuses, newborns, and immunocompromised individuals, with a 28% mortality rate (19, 38). This bacterium is ubiquitous on food surfaces and in food processing facilities, where it is able to survive for several months or even years (14, 36). It has been suggested that adhesion or biofilm formation is the main reason for the persistence of *L. monocytogenes* on food contact surfaces since bacterial cells within biofilms are much more resistant to detergents, biocides, and antibiotics than their free-living (planktonic) counterparts (10, 20). Consequently, *L. monocytogenes* biofilms on food contact surfaces are difficult to completely eliminate and may cause secondary food contamination, imposing major challenges to the production of safe food in the food industries (37).

Biofilms are highly organized communities, and their formation includes three steps: initial attachment, microcolony formation, and community expansion (5). Therefore, bacterial

cells within biofilms are metabolically different from their free-living forms. It is believed that extracellular signaling molecules (autoinducers of quorum sensing) are involved in regulating specific transcription factors, which then facilitate global controls in the bacterial genome expression, changing bacteria from a planktonic to a sessile lifestyle (15). Indeed, DNA microarray analysis has revealed that autoinducer 2 (AI-2) alters the expression of six transcription factors in *Escherichia coli* (7). Thus far, three quorum-sensing systems have been shown to function in bacterial cellular communication and to play roles in biofilm formation and maturation (1, 6). Firstly, acyl-homoserine lactones (AHLs) function as autoinducers of quorum sensing in the LuxI/LuxR signaling system found only in gram-negative bacteria (6, 13). Secondly, AI-2 signals of the LuxS communication system are used for quorum sensing in many distantly related bacteria such as the gram-negative *Salmonella* and the gram-positive *Staphylococcus* (34, 41). Thirdly, small peptides also function as quorum-sensing signals, exemplified by the *agr* system identified in *Staphylococcus aureus* and *L. monocytogenes* (15, 32, 41).

Recently, the biofilm-positive mutant LM-49 was isolated by screening a library constructed from *L. monocytogenes* 4b G using Tn917 mutagenesis. This mutant exhibited an enhanced ability to form biofilm compared to the wild-type strain (4). An elucidation of the function of the defective gene could lead to an improved understanding of biofilm formation of *L. monocytogenes*. Therefore, the purpose of this work was to characterize the functions of the mutant gene and to identify its mechanism for biofilm formation.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
<i>L. monocytogenes</i> strains		
4b G	Wild-type <i>L. monocytogenes</i> , isolate strain	Chinese Center for Disease Control, Hubei, China
LM-49	Enhanced biofilm-forming Tn917 mutant of <i>L. monocytogenes</i>	4
LM-49RE	Revertant <i>L. monocytogenes</i> strain from allelic recombination using pKSV7-AB	Sarah Vela
<i>E. coli</i> strains		
DH5 α	λ^- ϕ 80dlacZ Δ M15 Δ (lacZYA-argF)U196 recA1 endA1 hsdR17(r $_K^-$ m $_K^-$) supE44 thi-1 gyrA relA1	Laboratory stock
TG1	supE hsd Δ 5 thi Δ (lac-proAB) F' [traD36 proAB $^+$ lacI q lacZ Δ M15]	Laboratory stock
Plasmids		
pKSV7	Temperature-sensitive shuttle vector	This study
pKSV7-AB	Allelic recombination vector	This study

MATERIALS AND METHODS

Bacterial strains. The *L. monocytogenes* strain 4b G was obtained from the Chinese Center for Disease Control (Hubei, China). Serotyping of this isolate using a *Listeria* antisera kit (Denka Seiken Co., Tokyo, Japan) revealed that it belongs to the serotype 4b group. The biofilm-positive mutant LM-49 was generated from *L. monocytogenes* 4b G by Tn917 mutagenesis (4). The LM-49RE is a revertant of the LM-49 mutant in which the mutant allele lm.G1771::Tn917 is replaced with the wild-type gene via homologous recombination (see below).

L. monocytogenes 4b G and its derivatives were cultured in either Trypticase soy broth (TSB) medium (Merck, Germany) or brain heart infusion (BHI) medium (Becton, Dickinson, and Co., Franklin Lakes, NJ). To select cells containing Tn917, TSB or BHI medium was supplemented with 5 μ g/ml erythromycin (Erm), whereas the medium was supplemented with 10 μ g/ml chloramphenicol (Cam) to select cells harboring the *Listeria-E. coli* shuttle vector pKSV7 or its derivatives. The incubation temperature was 37°C for all *L. monocytogenes* strains used in this work, unless otherwise indicated specifically. *E. coli* DH5 α and TG1 were used as host strains for molecular cloning. All bacterial strains and plasmids used in this study are listed in Table 1.

Southern blot analysis. Genomic DNAs were prepared from *L. monocytogenes* 4b G and LM-49, and Southern blot analysis was done using standard procedures (30). In brief, the prepared genomic DNA was digested with EcoRI and run on a 1% agarose gel. The DNA fragments in the agarose gel were then transferred onto a Hybond-N $^+$ nylon membrane (GE Health Care) via capillary transfer. The probe for the Southern analysis was generated by PCR from a plasmid containing Tn917 by using primers Tn4292 and Tn3768 (Table 2). The PCR product was purified, labeled with biotin using a Gene Images Random Priming

DNA Labeling Kit (GE Health Care), and used as a probe for hybridization. Then, the hybridization signals on the membranes were detected using a Gene Image CDP-Star Detection kit (GE Health Care), following the instructions of the manufacturer, and recorded on Fuji X-ray film.

Identification of the Tn917 insertion site by inverse PCR. The DNA oligonucleotide primers used for PCRs were designed using the software Primer Premier, version 5 (33). To identify the target site of Tn917 in the mutant LM-49, two sets of primers were required, one for each flanking end (Fig. 1A). The primers C427 and Tn917F (Table 2) were used to clone the Tn917 5' flanking sequence, while primers Tn4431 and Tn5054 (Table 2) were used to obtain its 3' flanking sequence. An inverse PCR (iPCR) approach (22) was used since the primers in each set extend away from each other on a linear DNA template but face toward each other on a circular DNA template (Fig. 1A).

The genomic DNAs from the *L. monocytogenes* LM-49 and the wild-type *L. monocytogenes* 4b G (as negative control) strains were digested with HindIII at 37°C for 16 h in order to prepare the circular DNA used as a template for iPCR. The resulting DNA fragments were purified with a Nucleic Acid Purification kit (Qiagen) and ligated with T4 DNA ligase. The ligated circular DNA was then used as a template for iPCR and amplified using Ex-Tag DNA polymerase in the presence of 1 mM deoxynucleoside triphosphate mix. The amplification was done on a Peltier Thermal Cycler PTC225 (GMI, Ramsey, MN). The amplification conditions were as follows: an initial denaturation step at 95°C for 5 min; 34 cycles of 94°C for 30 s, followed by 50°C for 30 s, and 72°C for 2 min; and a final extension step at 72°C for 8 min. The PCR products were analyzed by agarose gel electrophoresis.

The purified PCR products were cloned into the PCR cloning vector

TABLE 2. Oligonucleotides used as primers

Primer name	Primer sequence	Location (bp)	Use
C427	5'-CTACATTACGCATTTGGA-3'	533–516 ^a	iPCR
Tn917F	5'-TGGTCTTAAGGCAGCCAGAG-3'	2180–2199 ^a	iPCR
Tn4431	5'-CCATCAAGGGTATGAGTA-3'	4537–4520 ^a	iPCR
Tn5054	5'-AAATAGATCCCGAAGTAAC-3'	5143–5161 ^a	iPCR
Tn4292	5'-TTGAGCACGAGTCAGAGC-3'	4292–4275 ^a	Southern blot probe
Tn3768	5'-AACAAATATTCGATCAGGAGA-3'	3767–3786 ^a	Southern blot probe
InsL	5'-AGCCATCAAAGAAAGGAAGA-3'	1794567–1794586 ^b	Amplification of insertion site
InsR	5'-GTATCGGTGAAAGGCAACAG-3'	1795615–1795596 ^b	Amplification of insertion site
LM DBR-F	5'-CGCTCAAACCAATTTCTGCTA-3'	1794114–1794134 ^b	RT-PCR
LM DBR-R	5'-CTTCCTTCTTTGATGGCTTT-3'	1794585–1794565 ^b	RT-PCR
16S rRNA-F	5'-TTAGTGGCGGACGGGTGAGT-3'	<i>rrsA-rrsB</i>	RT-PCR
16S rRNA-R	5'-CGGACAACGCTTGCCACCTA-3'	<i>rrsA-rrsB</i>	RT-PCR
P1	5'-GTCCGCCTTAATAGAGCA-3'	1794392–1794409 ^b	Identification of revertant
P2	5'-GTTCTACCGCTGGCTCTT-3'	1797225–1797208 ^b	Identification of revertant
C1	5'-GGGCGTCTCAGCTCTAAAATCCG-3'	4385–4406 ^a	Identification of allelic exchange
C2	5'-TAGGAACTATGAAAAGGAACGGA-3'	1729–1706 ^a	Identification of allelic exchange
M13fwd	5'-GGTTTTCCAGTCACGAC-3'		Identification of allelic exchange
M13rev	5'-AGCGGATAACAATTCACAC-3'		Identification of allelic exchange

^a Location in the published nucleotide sequence of Tn917 (accession number M11180).

^b Location in the published complete genome of *L. monocytogenes* strain 4b F2365 (accession number AE017262).

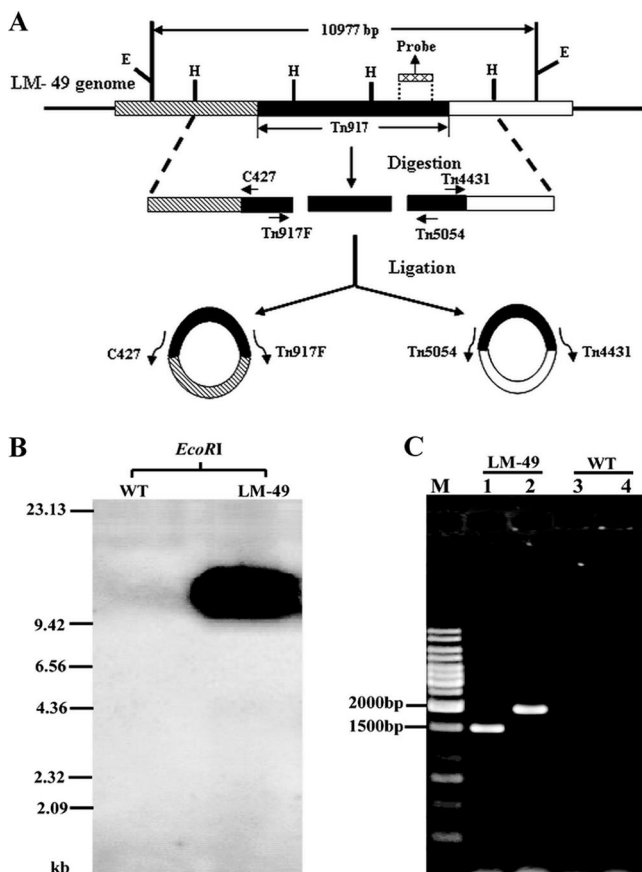


FIG. 1. Identification of the Tn917 insertion site in the *L. monocytogenes* LM-49 mutant. (A) Flow chart for generating DNA templates for iPCR: HindIII digestion of total DNA, circularization of the linear fragments by ligation, and iPCR. Two sets of primers were required for iPCR, one for each end since there are two HindIII (H) restriction sites in Tn917. E, EcoRI restriction sites. (B) Southern analysis of Tn917 insertion in the LM-49 mutant. The location of the probe in Tn917 is indicated in panel A. (C) Agarose gel electrophoresis of iPCR products. M, 1-kb DNA Ladder Marker; lanes 1 and 3, iPCR with C427 and Tn917F primers; lanes 2 and 4: iPCR with primers Tn4431 and Tn5054. LM-49 denotes DNA templates prepared from the mutant, and wild type (WT) denotes templates from *L. monocytogenes* 4b G (negative control).

pMD19-T (Takara Bio Inc.) following the procedure of the manufacturer, and the resulting plasmids were transformed into *E. coli* TG1 competent cells. The DNA sequences of both strands of the PCR products were then determined (Sangon Co. Ltd., Shanghai, China).

Construction of the plasmid pKSV7-AB for gene replacement and isolation of the revertant LM-49RE. A 1,049-bp DNA fragment that flanked the Tn917 insertion site was amplified from the wild-type *L. monocytogenes* 4b G genome by PCR, using primers InsL and InsR (Table 2). This PCR product was sequenced and cloned into pMD19-T, resulting in the plasmid pMD19-AB. The SalI-EcoRI fragment containing the PCR product was then cloned into the *Listeria-E. coli* shuttle plasmid pKSV7, yielding the plasmid pKSV7-AB for gene replacement.

pKSV7-AB was transformed into LM-49 competent cells via electroporation (25), and transformants harboring the plasmid were selected at 30°C in BHI medium containing 10 µg/ml Cam. Cells containing an integrated pKSV7-AB (pop-in recombinants) were selected by growth at 41°C (nonpermissive temperature for replication of this plasmid) for 40 generations. One pop-in recombinant was chosen and cultured in nonselective medium at 30°C for 30 generations. Under these conditions, all five types of recombinants could grow up, i.e., the strain containing the integrated pKSV7-AB after the first crossover and the four possible outcomes after the second allelic exchange, as illustrated in Fig. 3A. The

pop-out recombination types I and II containing plasmids pKSV7-Tn917 and pKSV7-AB, respectively, could grow on Cam plates at 30°C, while type III (revertant) and type IV (LM-49) with no plasmid could not grow on Cam plates. Several resulting strains were characterized by PCR, and a confirmed revertant strain, LM-49RE, was used for further experiments.

RNA preparation and reverse transcription-PCR (RT-PCR) analyses. *L. monocytogenes* 4b G and LM-49 cells were grown in BHI medium to an optical density at 600 nm (OD_{600}) of 0.7 for exponential phase samples and to 1.3 for stationary phase samples. Sessile cells from biofilms of both strains grown on glass slides were scraped off with cotton sticks, resuspended in 1 ml of 0.85% NaCl, and pelleted by centrifugation. The cells were resuspended in a 10 mg/ml solution of lysozyme and incubated at 37°C for 15 min to make the cell wall more permeable. Total RNA was extracted with Trizol reagent, following the manufacturer's procedure (Invitrogen). Six units of Turbo DNase I (Ambion) was used to treat 10 µg of RNA at 37°C for 2 h to ensure complete removal of residual DNA. DNase I was then extracted with phenol (pH 4.5), the RNA was precipitated with ethanol, and the RNA pellet was resuspended in 20 µl of H₂O pretreated with 0.1% diethyl pyrocarbonate.

cDNA was synthesized by RT-PCR in 10-µl reaction mixtures containing 100 ng of RNA, 2 pmol of RT-PCR primer (Table 2), 10 U of RNase inhibitor, and 25 U of Moloney murine leukemia virus reverse transcriptase (Ambion), and the mixture was incubated at 42°C for 30 min. One unit of HotStart *Taq* DNA Polymerase (Qiagen, Germany) was used for RT-PCRs in the presence of 1 mM deoxynucleoside triphosphate mix in a 25-µl reaction system.

For analysis of *lm.G_1770* expression, three samples of each cDNA preparation were amplified for 32, 35, and 38 cycles with conditions of 94°C for 30 s, 55°C for 30 s, and 72°C for 40 s. The PCRs for 16S rRNA expression were 22, 25, and 28 cycles with conditions of 94°C for 30 s, 63°C for 30 s, and 72°C for 1 min. The synthesized DNA was analyzed by agarose gel electrophoresis.

Biofilm formation assay. Biofilm formation by *L. monocytogenes* was monitored using the microplate assay described by Djordjevic et al. (8). In brief, *L. monocytogenes* cells were inoculated in 96-well microplates and cultured at 37°C for 48 h. Free-moving (planktonic) cells in the liquid medium were removed by gentle pipetting, while sessile cells in the biofilms remained attached to the wells of the plate. To estimate the cell mass of the biofilms, 1% crystal violet was added to each well to stain the cells. After the unbound dye was removed and plates were rinsed with water, the crystal violet bound to the sessile cells in the biofilms was recovered by ethanol extraction, and the amount was determined by measuring the absorbance at OD_{595} with a DU-800 spectrophotometer (Beckman Instruments). The biomass of a biofilm was expressed as the amount of violet crystal bound to the plates. To determine the number of attached cells in the biofilms, the sessile cells were scraped off from the wells of microplates using sterile cotton sticks and resuspended. Serial decimal dilutions were then made with 0.85% NaCl solution, and the diluted cells were spread on BHI plates. This procedure was repeated three times for each sample to assess the reproducibility of the data.

Fluorescence microscopy of biofilm. Samples for fluorescence microscopy were prepared on glass slides using a method described by Marsh et al. (17) with the modification that fluorescein isothiocyanate (FITC) (21) was used instead of acridine orange. Briefly, petri dishes containing 20 ml of TSB medium and a sterilized glass slide were inoculated with *Listeria* cells and incubated at 37°C for 72 h, during which time *Listeria* cells formed biofilms on the glass slide. The slide was then rinsed three times with sterile water to remove all planktonic cells, air dried, and immersed in 0.1% FITC (Sigma-Aldrich) for 15 to 20 min at room temperature in the dark to stain the sessile cells in the biofilms. FITC was poured off, excess dye was removed by rinsing the slide three times with water, and the biofilms formed were examined with the computer-assisted Olympus fluorescence microscope BX51 (Olympus company, Japan) and photographed.

Nucleotide sequence accession number. The entire nucleotide sequence of *lm.G_1771* was deposited in the GenBank database under accession number FJ216726.

RESULTS

Tn917 transposition inactivated the gene encoding a putative ABC transporter permease in LM-49. Previously, an *L. monocytogenes* mutant, LM-49, exhibiting enhanced biofilm formation (biofilm-positive mutant) was isolated from a mutant library generated by Tn917 mutagenesis (4). We chose to investigate the mechanism of the biofilm formation in *Listeria* using this mutant. To ascertain that LM-49 was generated from

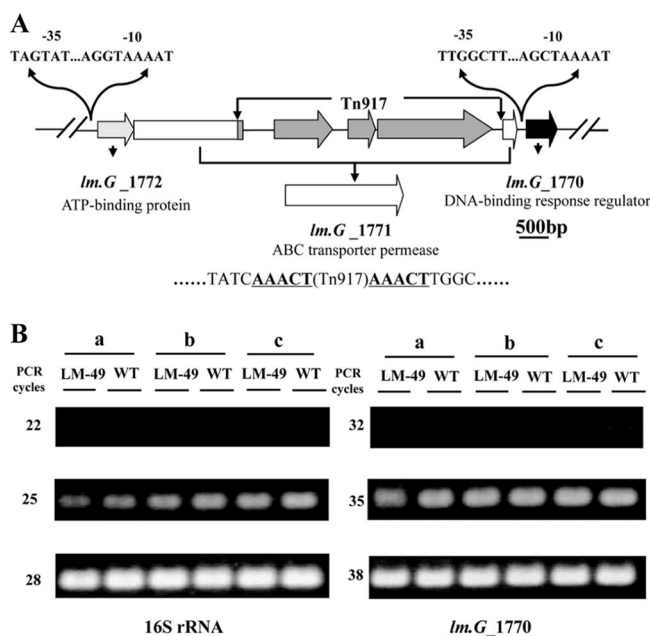


FIG. 2. (A) Genetic map of the *lm.G_1771::Tn917* mutant allele. Putative promoter elements identified upstream of *lm.G_1772* and *lm.G_1770* are indicated. A typical 5-bp target sequence duplicated by *Tn917* transposition is indicated in boldface letters and underlined. (B) Effect of *Tn917* insertion on the expression of downstream gene. a, exponential growth phase; b, stationary growth phase; c, sessile growth phase (biofilms). WT, the wild-type strain; LM-49, the biofilm-positive mutant. PCR with the purified RNA as a template did not yield any product (data not shown). This experiment was repeated three times with similar results.

a single *Tn917* transposition event, a *Tn917*-specific sequence (Fig. 1A) was amplified by PCR, labeled with biotin, and used as a probe in Southern blot analysis of the mutant and wild-type DNA (Materials and Methods). As shown in Fig. 1B, the *Tn917* probe specifically recognized only one hybridized fragment from the LM-49 genome and no hybridized signal from the *L. monocytogenes* 4b G wild type. These results indicated that the LM-49 mutant carried only one copy of *Tn917*.

Next, the LM-49 gene harboring the *Tn917* insertion was identified by iPCR since the *Tn917* sequence is known. As illustrated in Fig. 1A, oppositely oriented *Tn917* primers point toward each other on the circularized DNA, and this enabled the *Tn917*-flanking sequence of the host chromosome, including the target site of the *Tn917* transposition, to be cloned. The key step in iPCR is to generate suitable circular DNA molecules. Hence, the *Hind*III restriction enzyme (that cuts twice in *Tn917*) was used to cleave the LM-49 genomic DNA, yielding linear DNA fragments. These fragments were ligated with T4 DNA ligase, resulting in circular DNA substrates for iPCR (Fig. 1A). Two specific PCR products of 1.5 and 2.0 kb were obtained, one for each primer set (Fig. 1C). The 1.5-kb fragment comprised the *Tn917* upstream-flanking fragment, while the 2.0-kb fragment contained the *Tn917* downstream-flanking sequence.

The two PCR fragments were cloned into the pMD19-T vector and sequenced. Analyses of the sequences obtained indicated the following: (i) the target gene encoded a putative

ABC transporter permease of 659 amino acids, which was 100% identical to the permease encoded by *Lmof2365_1771*; (ii) the insertion point was at 1,715 bp after the ATG start codon of the gene; (iii) the transposon was oriented in the same transcriptional direction as the target gene; and (iv) the 5-bp target sequence (5'-AAACT-3') was duplicated (Fig. 2A). We named this gene *lm.G_1771*, referring to its homology with *Lmof2365_1771* present in *L. monocytogenes* strain 4b F2365, and we named the mutant allele in LM-49 *lm.G_1771::Tn917*.

***Tn917* insertion did not change transcription of a downstream gene and the phenotype in biofilm formation.** In addition to mutagenesis by transposition into target genes, *Tn917* mutagenesis may produce polar effects on the expression of downstream genes in the mutant by introducing transcriptional termination and/or changing transcriptional readthrough (9, 16). To study this possible polar effect, we used semiquantitative RT-PCR to analyze the expression of *lm.G_1770* immediately downstream of the *lm.G_1771* gene in *L. monocytogenes* 4b G and in LM-49 (Fig. 2A). As shown in Fig. 2B, the expression of *lm.G_1770* in the wild-type and mutant strains remained the same for cells from exponential, stationary, and sessile growth phases. Thus, inactivation of the ABC transporter permease gene *lm.G_1771* by *Tn917* transposition did not have a polar effect on the downstream gene. Furthermore, sequence analysis of the promoter region of *lm.G_1770* revealed a putative promoter 33 bp upstream of the ATG start codon (Fig. 2A). Thus, the expression of this gene was very likely only from this promoter.

To exclude the possibility that the *Tn917* insertion element influences biofilm formation, we also constructed a deletion mutant. This mutant had the same phenotype for biofilm formation as LM-49 (data not shown).

Construction of a revertant of LM-49 by replacing *lm.G_1771::Tn917* with the wild-type gene *lm.G_1771*. To demonstrate that the *Tn917* insertion in *lm.G_1771* (*lm.G_1771::Tn917*) was solely responsible for the enhanced biofilm formation of the mutant, a revertant was constructed by replacing *lm.G_1771::Tn917* with the wild-type gene *lm.G_1771* via homologous recombination (Fig. 3A).

The temperature-sensitive plasmid pKSV7-AB, which contains the *lm.G_1771* fragment, was transformed into LM-49. The transformation mixtures were spread onto Cam-containing plates to select for Cam^r transformants at a permissible temperature (30°C). A transformant was chosen and grown at the nonpermissible temperature of 41°C, thereby forcing integration of the plasmid into the chromosome to yield the pKSV7-AB pop-in recombinants. About 30 colonies were randomly picked and analyzed by PCR to identify *Tn917* and pKSV7 junctions that were characteristic for the two possible types of pKSV7-AB pop-in (Fig. 3A, panel 1). A PCR product of the expected size was obtained from amplification with primers M13fwd and C1 but not with M13rev and C2 (Fig. 3B), suggesting that site "a" was preferred to site "b" in the pop-in recombination.

To obtain revertants, one purified pop-in recombinant (LM-49::pKSV7-AB) was grown for 30 generations at 30°C. Approximately 700 colonies were replica plated onto plates containing either Erm or Cam and incubated at 30°C. Colonies with type I and type II recombinations grew on both antibiotic-containing plates since they contained plasmids pKSV7-*Tn917*

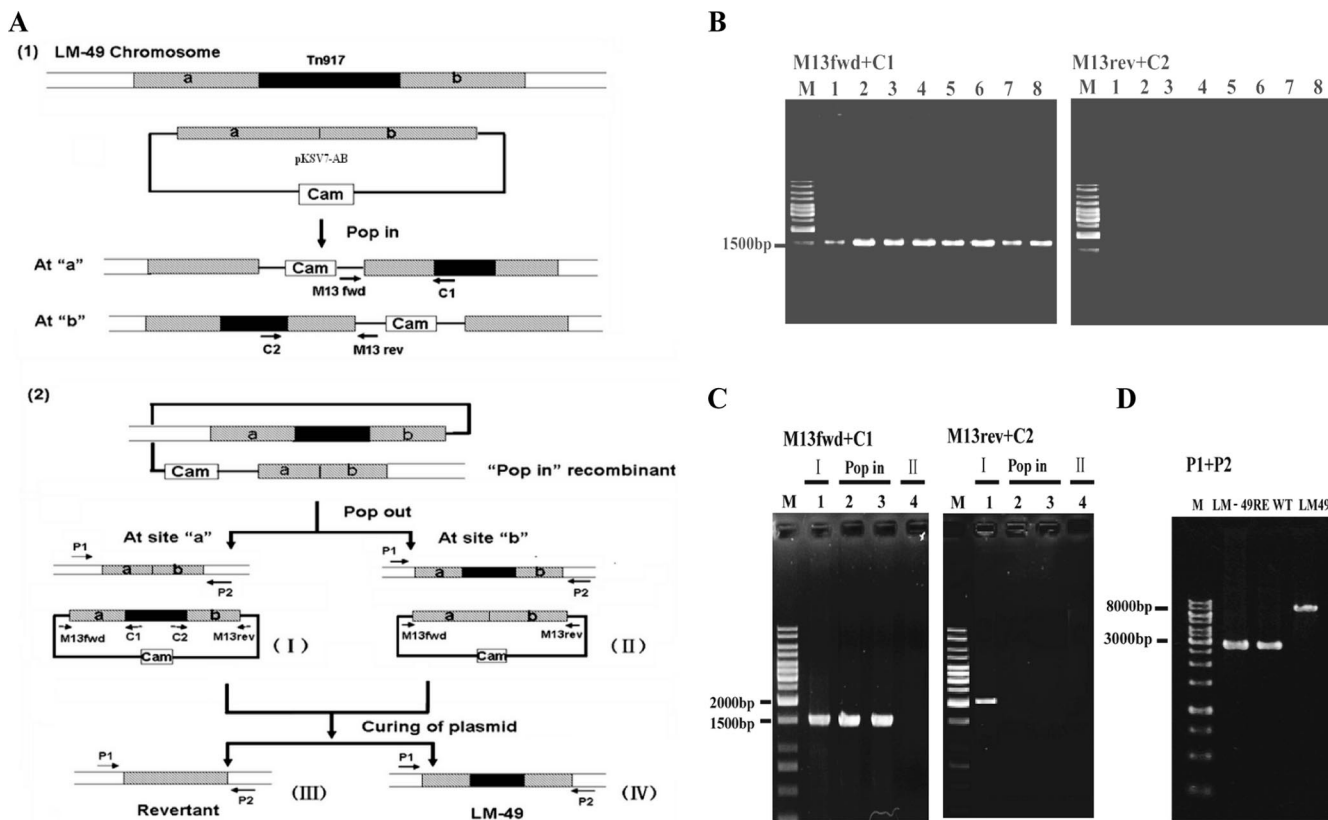


FIG. 3. Construction of the LM-49 revertant via homologous recombination. (A) Flow chart of LM-49 revertant construction (1). In the LM-49 strain harboring the gene replacement plasmid pKSV7-AB, the plasmid may integrate into the host chromosome at either site a or site b (Pop in) (2). Four possible outcomes of pop-out recombination are shown. The PCR primer sets P1+P2, M13fwd+C1, and M13rev+C2 were used for distinguishing between these recombinations by PCR. (B) Identification of pKSV7-AB integration by colony PCR. A total of 30 colonies were purified and tested. The results from eight of them are shown. M, 1-kb DNA ladder marker; lanes 1 to 8, purified transformants PCR checked with M13fwd+C1 or M13rev+C2 as primers. (C) Analysis of plasmid and Tn917 junctions for checking type I and II colonies by colony PCR. (D) PCR identification of the revertant LM-49RE.

and pKSV7-AB, respectively. Colonies with type IV recombination (LM-49) grew on Erm plates but not on Cam plates. Finally, pop-out revertants (type III) grew on neither Erm nor Cam plates. To further confirm the pop-out event, PCR amplification of plasmid and Tn917 junctions was performed to distinguish between type I and II colonies (Fig. 3A, panel 2). As expected, both M13fwd+C1 and M13rev+C2 primers yielded a PCR product for type I recombination and none for type II (Fig. 3C). Furthermore, similar PCR fragments of *lm.G_1771* were obtained from a revertant (LM-49RE) and from the *L. monocytogenes* 4b G wild type using the primers P1 and P2 (Fig. 3D). The same results were obtained for the 18 revertants found (data not shown).

Conservation of the *Lm.G_1771* ABC transporter permease and related gene products among gram-positive bacteria. A search for sequence homologues of *Lm.G_1771* permease in the GenBank database revealed that the *Lm.G_1771* permease is highly conserved in *Listeria* species; its homologues were found in all sequenced *Listeria* genomes showing more than 95% sequence identity. Structural analysis of the *Lm.G_1771* protein sequence further revealed that it contained 10 transmembrane fragments (<http://www.cbs.dtu.dk/services/TMHMM/>). Moreover, genes *lm.G_1771* and *lm.G_1772* (which encodes a

putative ATP-binding protein) were clustered, and they most likely form an operon (Fig. 2A). These results suggest that the *Lm.G_1771* permease and *Lm.G_1772* ATP-binding protein constitute an uncharacterized ABC transporter.

Furthermore, a similar ABC transporter was found to be encoded by other bacterial genomes as well. By searching the public databases with the protein sequences of *Lm.G_1771* and *Lm.G_1772*, several open reading frames with significant similarity to the components of the putative transporter were found, one of which is a known drug efflux protein that exports bacitracin (24). They all comprise two components, the permease and the ATP-binding protein, and it appears that the genes encoding them are also organized into operons (Fig. 4). The sequence similarity between the identified *Listeria* ABC transporter and the *Bacillus* bacitracin drug efflux protein was the highest among these drug efflux proteins: 26% sequence identity between their permease components (*Lm.G_1771* versus *BecB*) and 54% identity for the ATP-binding components (*Lm.G_1772* versus *BecA*). Thus, *Lm.G_1771* and *Lm.G_1772* are likely to encode an export ABC transporter. Preliminary data, however, do not indicate that bacitracin susceptibility has changed in the LM-49 mutant (data not shown). In the follow-

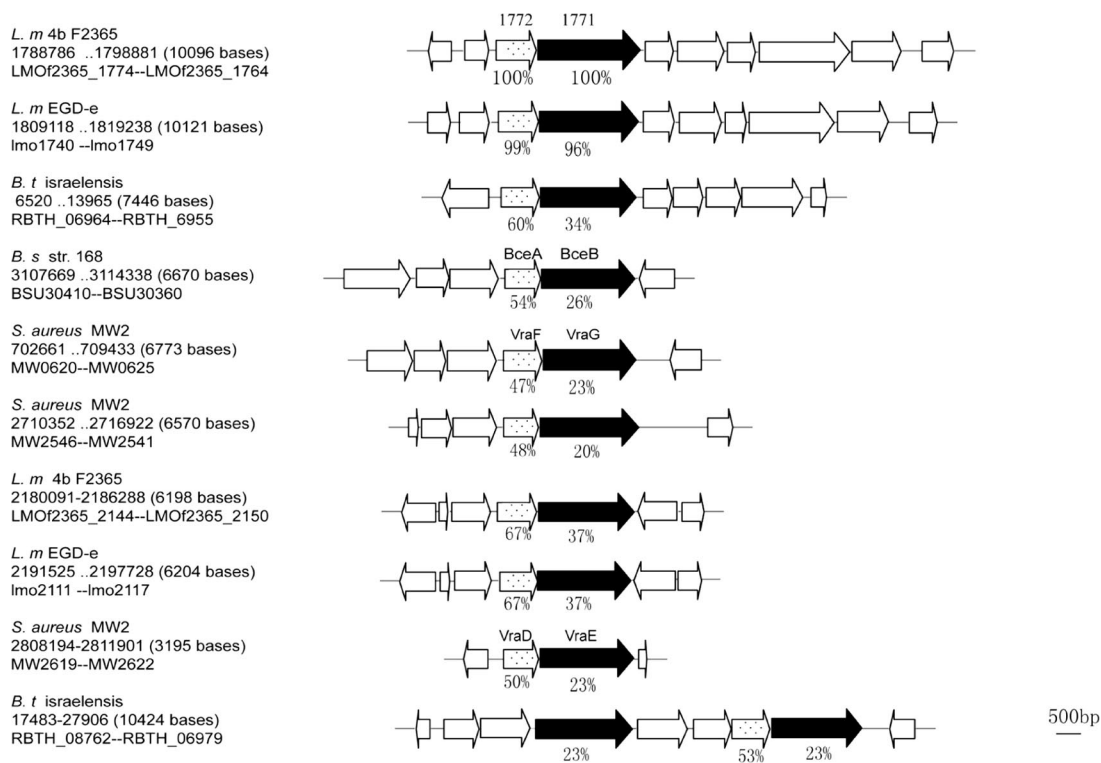


FIG. 4. Organization of the putative Lm.G_1771 ABC transporter and its homologous systems identified in bacterial genomes. The filled boxes indicate the homologues of the putative Lm.G_1771 permease, and the dotted boxes show the open reading frames encoding an ATP-binding protein. The percentages under the gene symbols indicate the sequence identities between Lm.G_1771, Lm.G_1772, and their homologues. The homologues were identified by BlastP searches (27) and aligned using the Clustal W program (12). *L. m* 4b F2365, *L. monocytogenes* strain 4b F2365; *L. m* EGD-e, *L. monocytogenes* EGD-e; *B. t* israelensis, *Bacillus thuringiensis* serovar israelensis; *B. s* str.168, *Bacillus subtilis* subsp. *subtilis* strain 168; *S. aureus* MW2, *S. aureus* subsp. *aureus* MW2.

ing, we call this putative ABC transporter the Lm.G_1771 transporter.

Lm.G_1771 ABC transporter permease as a negative regulator of biofilm formation by *Listeria*. Biofilm formation by *L. monocytogenes* 4b G and its biofilm-positive mutant LM-49 and corresponding revertant LM-49RE was studied using microplate assay and fluorescence microscopy. As seen in Fig. 5A, the LM-49RE revertant and the wild-type strain produced similar amounts of biofilm, while the LM-49 mutant formed a thicker biofilm layer. Quantification of the biomass using crystal violet staining also showed that the LM-49 mutant formed more biofilm than either the revertant or parental strain (Fig. 5A). Furthermore, fluorescence microscopy showed that the biofilms formed by these strains exhibited distinct morphologies; the wild-type strain and the revertant LM-49RE showed the same tread-like pattern, whereas large cell aggregates were built up along the treads in the LM-49 biofilm (Fig. 5B). These results suggest that the Lm.G_1771 transporter could be an integral part of signal transduction participating in the negative regulation of biofilm formation in *Listeria*.

The Lm.G_1771 transporter may export a signal inhibiting biofilm formation. The enhanced biofilm-forming ability of LM-49 would be expected if the mutant cells were defective in exporting a signal inhibiting biofilm formation. Therefore, if wild-type and mutant cells were to be mixed, their ability to form biofilm should now be similar, since all cells would be

exposed to the signal from the wild-type cells. On the other hand, if the mutant cells were not defective in exporting a signaling molecule, there would be no signal in the extracellular environment to inhibit biofilm formation in the mixed culture, and then the ratio of LM-49 to wild-type cells would be higher in the biofilms since the mutant itself has higher ability of biofilm formation than the wild type.

To test this hypothesis, we mixed wild-type and mutant cells at a 1:1 ratio in triplicate and incubated the mixture in a microplate at 37°C for 48 h, allowing biofilms to form. Pure cultures of the wild-type and mutant strains were incubated similarly.

The biofilm biomass in all cultures and the number of wild-type and mutant cells in the biofilms from mixed culture were determined by crystal violet quantification and counting CFU on plates (the mutant is *Erm*^r), respectively. The mutant LM-49 produced approximately twofold more biofilm mass than the wild type in pure culture (Fig. 6), and the ratio of wild-type to mutant cells in the mixed culture basically remained unchanged (52% wild-type cells versus 48% mutant cells). The growth rate and the maximum OD in stationary phase were also essentially the same for both the wild-type and mutant strains (data not shown). The results of this experiment are in agreement with our notion that, in contrast to the wild-type strain, the mutant LM-49 strain is unable to export a signal inhibiting biofilm formation.

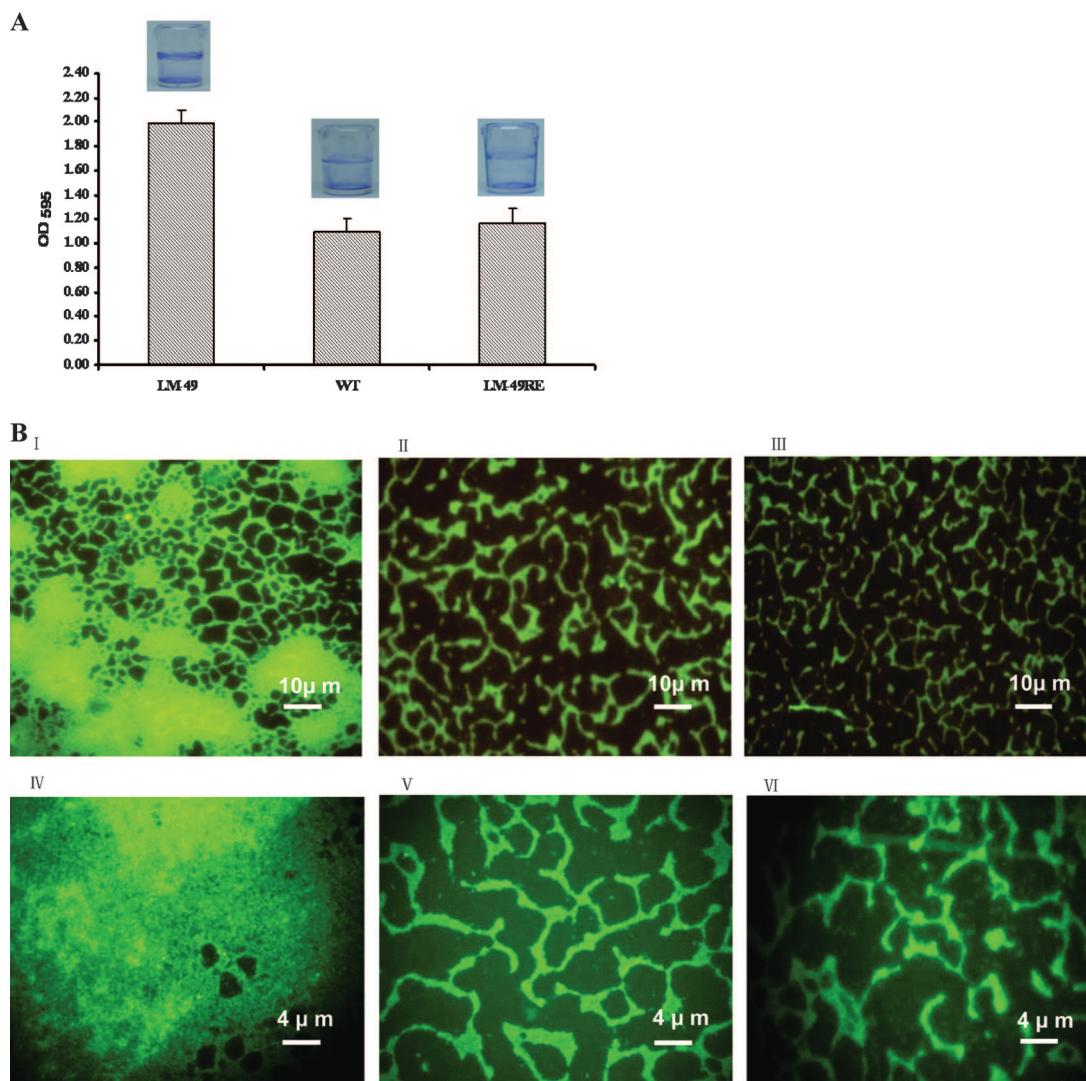


FIG. 5. Characterization of the biofilms produced by different *L. monocytogenes* strains. (A) Biomass quantification using crystal violet staining. Pictures of biofilm formed in microplates are shown above each column. (B) Fluorescence micrograph. Pictures in panels I and IV represent the biofilm formed by the LM-49 mutant; panels II and V represent the wild type (WT), and panels III and VI represent the revertant LM-49RE. Upon incubation on glass slides for 72 h, *L. monocytogenes* formed biofilms.

DISCUSSION

In this study, we have identified a new transporter that is involved in negative regulation of biofilm formation by *L. monocytogenes*. When one of the components of the ABC transporter was inactivated by a mutation, the resulting *L. monocytogenes* LM-49 mutant exhibited a stronger capacity for biofilm formation. Since biofilm formation is a complex process causing major physiological changes, the process could be regulated by global transcriptional factors that, in turn, respond to a signaling molecule such as a quorum-sensing signal. Therefore, the Lm.G_1771 ABC transporter may be an efflux protein exporting a signaling molecule that activates a pattern of genome expression characteristic of planktonic growth of *L. monocytogenes*.

A typical ABC transporter consists of a few subunits and contains one of the following structural domains: a nucleotide-

binding domain, a transmembrane domain, or a solute-binding domain (SBD) (35). All ABC transporters are either exporters or importers. Specifically, importers harbor at least one SBD, whereas exporters have no SBD (28). Although Lm.G_1771 encodes a permease with 10 transmembrane fragments and Lm.G_1772 encodes an ATP-binding protein, none of the neighboring genes encodes an SBD (Fig. 4), which suggests that the encoded ABC transporter is an exporter. Furthermore, it is very likely that this ABC transporter operon consists of only two genes, Lm.G_1772 and Lm.G_1771, since they share a common promoter (Fig. 2A). The immediate downstream gene, Lm.G_1770, encoding a putative DNA-binding response regulator, does not belong to the putative operon since it has its own promoter (Fig. 2A). In fact, as seen from semiquantitative RT-PCR analyses, its expression levels did not change in the wild-type and LM-49 strains (Fig. 2B).

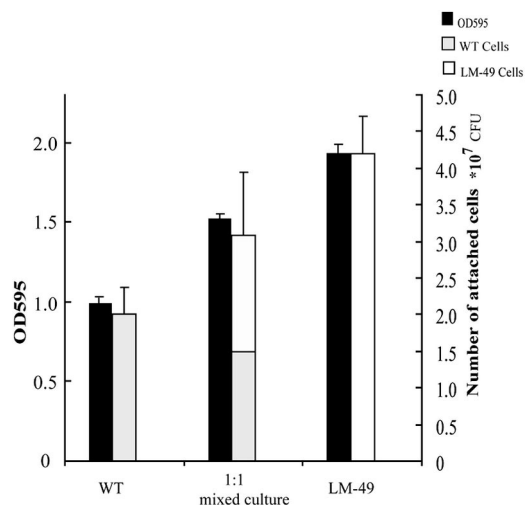


FIG. 6. Characterization of signal transport in the LM-49 mutant. Biofilms formed by pure cultures of the wild type (WT) and LM-49 and by a culture containing a 1:1 mixture thereof. The biofilm biomasses were estimated using crystal violet staining and viable cell determination.

Lm.G_1771 may represent an unknown transporter in *Listeria* because all known *Listeria* genomes encode such a transporter with high sequence similarity (>95%) while Lm.G_1771 shows only 26% sequence identity to BceB (YtsD) in *Bacillus subtilis*, the closest homologue of the permease among those identified in non-*Listeria* bacterial species. The LM-49 and its parent wild-type strain exhibited the same level of resistance to bacitracin (data not shown), which further indicates that the permease function differs from that of the *Bacillus* bacitracin drug efflux protein (23).

However, it has been established that several transporters are responsible for transporting signaling molecules that regulate bacterial biofilm formation. These chemical signals include AHL, the furanone derivative AI-2, and signal peptides. Although it has been suggested that small signal molecules can diffuse freely across the cellular membrane, active transport is also involved in exporting small signals. For example, while the AHL signals *N*-3-oxo-hexanoyl-homoserine lactone (HSL) and *N*-butyryl-HSL are freely diffusible, *N*-3-oxo-dodecanoyl-HSL has to be exported by the MexAB-OprM active efflux pump (26). Furthermore, for bacteria using AI-2 signals for quorum sensing to regulate their bioluminescence and biofilm formation (2, 18), the AI-2 molecules diffuse freely across the cell membrane in some bacteria and are detected by a two-component regulatory system as in *Vibrio harveyi* (31), while in other bacteria the AI-2 signals are imported into the cell via an *lsr* ABC transporter as in *Salmonella enterica* serovar Typhimurium and *E. coli* (40). With large signaling molecules such as signal peptides, active transport is always responsible for both exporting and importing, as in the case of the *agr* signaling system of *S. aureus* where the autoinducer peptide is exported by the AgrB transporter (39). These transporters are integral parts of their corresponding signaling systems. Therefore, inactivating any of these signal transporters should abolish the corresponding signal transmission and, consequently, terminate the programmed regulation of genome expression. As for

the LM-49 mutant, mutation in the Lm.G_1771 transporter has resulted in the derepression of biofilm formation, leading to the observed biofilm-positive phenotype. However, the Lm.G_1771 transporter does not exhibit sequence similarity to any known transporters of signaling molecules. It could, therefore, represent a novel exporter for chemical signals in bacteria.

The LM-49 mutant and the wild-type strain were cocultured at a 1:1 ratio for a biofilm formation assay using microplates. The biofilms of the mixed culture as well as the composition of the cells in the biofilms were analyzed. Interestingly, we found in the biofilms formed by the mixed culture that the ratio of mutant to wild-type cells did not change, suggesting that the wild-type cells produced a signal molecule and were able to inhibit biofilm formation in the LM-49 mutant cells. Therefore, we postulate that the *L. monocytogenes* LM-49 mutant may be defective in exporting a signal that leads to the inhibition of biofilm formation in *L. monocytogenes*. Consequently, the Lm.G_1771 transporter could be an exporter of this signal.

It remains to be determined which signal is exported by the Lm.G_1771 transporter. Currently, two quorum-sensing signaling systems are known in *L. monocytogenes*: the *agr* peptide and the AI-2 signals (2, 23, 26). Clearly, the Lm.G_1771 transporter cannot be responsible for transporting an *agr* signal since the complete pathway of the *agr* signal transduction has been identified in this bacterium (29). However, it is not clear whether this transporter transports an AI-2 signal. Genetic analyses of *luxS*, the gene encoding the enzyme for AI-2 biosynthesis, have indicated that AI-2 functions as an inhibitor of biofilm formation in *L. monocytogenes* EGD-e (3, 32), and it has been reported that defects in AI-2 biosynthesis has resulted in more than a 17-fold increase in biofilm formation (3). The Lm.G_1771 transporter could constitute an integral part of the AI-2 signaling system since some AI-2 molecules rely on an exporter for export into the extracellular environments (11). If so, LM-49 could be important in further elucidation of the AI-2 signaling system. Alternatively, the Lm.G_1771 transporter may export a novel signal.

In conclusion, we have shown that the *lm.G_1771* gene encoded a putative ABC transporter permease. When *lm.G_1771* was inactivated, it caused enhanced biofilm-formation of *L. monocytogenes*. The upstream gene, *lm.G_1772*, encodes an ATP binding protein, and the products of these two genes may form a putative ABC transporter. We also found that the sequence of this ABC transporter was highly conserved in *Listeria* species. Finally, the ratio of wild-type to mutant cells in the biofilm formed by a mixed culture was more or less constant, suggesting that this ABC transporter most likely exported a signal inhibiting biofilm formation of *L. monocytogenes*. Further investigation is required to elucidate precisely how the ABC transporter controls *L. monocytogenes* biofilm formation.

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REFERENCES

- Abraham, W. R. 2006. Controlling biofilms of gram-positive pathogenic bacteria. *Curr. Med. Chem.* **13**:1509–1524.
- Bassler, B. L., M. Wright, and M. R. Silverman. 1994. Multiple signalling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway. *Mol. Microbiol.* **13**:273–286.
- Challan Belval, S., L. Gal, S. Margiewes, D. Garmyn, P. Piveteau, and J. Guzzo. 2006. Assessment of the roles of LuxS, S-ribosyl homocysteine, and autoinducer 2 in cell attachment during biofilm formation by *Listeria monocytogenes* EGD-e. *Appl. Environ. Microbiol.* **72**:2644–2650.
- Chen, Y. H., and X. M. Shi. 2005. Mutagenesis on biofilm formation of *Listeria monocytogenes* by Tn917 transposon insertion. *Wei Sheng Wu Xue Bao* **45**:952–954.
- Costerton, J. W., P. S. Stewart, and E. P. Greenberg. 1999. Bacterial biofilms: a common cause of persistent infections. *Science* **284**:1318–1322.
- Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* **280**:295–298.
- DeLisa, M. P., C. F. Wu, L. Wang, J. J. Valdes, and W. E. Bentley. 2001. DNA microarray-based identification of genes controlled by autoinducer 2-stimulated quorum sensing in *Escherichia coli*. *J. Bacteriol.* **183**:5239–5247.
- Djordjevic, D., M. Wiedmann, and L. A. McLandsborough. 2002. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Appl. Environ. Microbiol.* **68**:2950–2958.
- Dobinsky, S., K. Bartscht, and D. Mack. 2002. Influence of Tn917 insertion on transcription of the *icaADBC* operon in six biofilm-negative transposon mutants of *Staphylococcus epidermidis*. *Plasmid* **47**:10–17.
- Frank, J. F., and R. A. Koffi. 1990. Surface-adherent growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitizers and heat. *J. Food Prot.* **53**:550–554.
- Herzberg, M., I. K. Kaye, W. Peti, and T. K. Wood. 2006. YdgG (TqsA) controls biofilm formation in *Escherichia coli* K-12 through autoinducer 2 transport. *J. Bacteriol.* **188**:587–598.
- Higgins, D. G., J. D. Thompson, and T. J. Gibson. 1996. Using CLUSTAL for multiple sequence alignments. *Methods Enzymol.* **266**:383–402.
- Juhas, M., L. Eberl, and B. Tummeler. 2005. Quorum sensing: the power of cooperation in the world of *Pseudomonas*. *Environ. Microbiol.* **7**:459–471.
- Kathariou, S. 2002. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. *J. Food Prot.* **65**:1811–1829.
- Kong, K. F., C. Vuong, and M. Otto. 2006. *Staphylococcus* quorum sensing in biofilm formation and infection. *Int. J. Med. Microbiol.* **296**:133–139.
- Le Coq, D., S. Aymerich, and M. Steinmetz. 1991. Dual effect of a Tn917 insertion into the *Bacillus subtilis* *sacX* gene. *J. Gen. Microbiol.* **137**:101–106.
- Marsh, E. J., H. Luo, and H. Wang. 2003. A three-tiered approach to differentiate *Listeria monocytogenes* biofilm-forming abilities. *FEMS Microbiol. Lett.* **228**:203–210.
- McNab, R., S. K. Ford, A. El-Sabaeny, B. Barbieri, G. S. Cook, and R. J. Lamont. 2003. LuxS-based signaling in *Streptococcus gordonii*: autoinducer 2 controls carbohydrate metabolism and biofilm formation with *Porphyromonas gingivalis*. *J. Bacteriol.* **185**:274–284.
- Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* **5**:607–625.
- Mustapha, A., and M. B. Liewen. 1989. Destruction of *Listeria monocytogenes* by sodium hypochlorite and quaternary ammonium sanitizers. *J. Food Prot.* **52**:306–311.
- Nielsen, A. T., T. Tolker-Nielsen, K. B. Barken, and S. Molin. 2000. Role of commensal relationships on the spatial structure of a surface-attached microbial consortium. *Environ. Microbiol.* **2**:59–68.
- Ochman, H., J. W. Ajioka, D. Garza, and D. L. Hartl. 1990. Inverse polymerase chain reaction. *Nat. Biotechnol.* **8**:759–760.
- Ohki, R., Giyanto, K. Tateno, W. Masuyama, S. Moriya, K. Kobayashi, and N. Ogasawara. 2003. The BceRS two-component regulatory system induces expression of the bacitracin transporter, BceAB, in *Bacillus subtilis*. *Mol. Microbiol.* **49**:1135–1144.
- Ohki, R., K. Tateno, Y. Okada, H. Okajima, K. Asai, Y. Sadaie, M. Murata, and T. Aiso. 2003. A bacitracin-resistant *Bacillus subtilis* gene encodes a homologue of the membrane-spanning subunit of the *Bacillus licheniformis* ABC transporter. *J. Bacteriol.* **185**:51–59.
- Park, S. F., and G. S. Stewart. 1990. High-efficiency transformation of *Listeria monocytogenes* by electroporation of penicillin-treated cells. *Gene* **94**:129–132.
- Pearson, J. P., C. Van Delden, and B. H. Iglewski. 1999. Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. *J. Bacteriol.* **181**:1203–1210.
- Pearson, W. R. 1995. Comparison of methods for searching protein sequence databases. *Protein Sci.* **4**:1145–1160.
- Quentin, Y., G. Fichant, and F. Denizot. 1999. Inventory, assembly and analysis of *Bacillus subtilis* ABC transport systems. *J. Mol. Biol.* **287**:467–484.
- Saenz, H. L., V. Augsburger, C. Vuong, R. W. Jack, F. Gotz, and M. Otto. 2000. Inducible expression and cellular location of AgrB, a protein involved in the maturation of the staphylococcal quorum-sensing pheromone. *Arch. Microbiol.* **174**:452–455.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schauder, S., and B. L. Bassler. 2001. The languages of bacteria. *Genes Dev.* **15**:1468–1480.
- Sela, S., S. Frank, E. Belausov, and R. Pinto. 2006. A mutation in the *luxS* gene influences *Listeria monocytogenes* biofilm formation. *Appl. Environ. Microbiol.* **72**:5653–5658.
- Singh, V. K., A. K. Mangalam, S. Dwivedi, and S. Naik. 1998. Primer premier: program for design of degenerate primers from a protein sequence. *BioTechniques* **24**:318–319.
- Taga, M. E., J. L. Semmelhack, and B. L. Bassler. 2001. The LuxS-dependent autoinducer AI-2 controls the expression of an ABC transporter that functions in AI-2 uptake in *Salmonella typhimurium*. *Mol. Microbiol.* **42**:777–793.
- Tam, R., and M. H. Saier, Jr. 1993. Structural, functional, and evolutionary relationships among extracellular solute-binding receptors of bacteria. *Microbiol. Rev.* **57**:320–346.
- Tompkin, R. B. 2002. Control of *Listeria monocytogenes* in the food-processing environment. *J. Food Prot.* **65**:709–725.
- Trachoo, N. 2003. Biofilms and the food industry. *Songklanakaraj J. Sci. Technol.* **25**:807–815.
- Vazquez-Boland, J. A., M. Kuhn, P. Berche, T. Chakraborty, G. Dominguez-Bernal, W. Goebel, B. Gonzalez-Zorn, J. Wehland, and J. Kreft. 2001. *Listeria* pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev.* **14**:584–640.
- Vuong, C., H. L. Saenz, F. Gotz, and M. Otto. 2000. Impact of the *agr* quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. *J. Infect. Dis.* **182**:1688–1693.
- Walters, M., and V. Sperandio. 2006. Quorum sensing in *Escherichia coli* and *Salmonella*. *Int. J. Med. Microbiol.* **296**:125–131.
- Xu, L., H. Li, C. Vuong, V. Vadyvaloo, J. Wang, Y. Yao, M. Otto, and Q. Gao. 2006. Role of the *luxS* quorum-sensing system in biofilm formation and virulence of *Staphylococcus epidermidis*. *Infect. Immun.* **74**:488–496.