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A *mariner*-Based Transposition System for *Listeria monocytogenes*▽

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In this study, we developed a new *mariner*-based transposition system for *Listeria monocytogenes*. The *mariner*-based system has a high rate of transposition and a low rate of plasmid retention, and transposition is very random, making it an ideal tool for high-throughput transposon mutagenesis in *L. monocytogenes*.

Listeria monocytogenes is a saprophytic gram-positive bacterial rod that is ubiquitous in nature and is an opportunistic foodborne pathogen of humans and a variety of other vertebrates (24). During infection, *L. monocytogenes* multiplies intracellularly in the cytosols of host cells (19, 23). Intracellular survival of *L. monocytogenes* relies largely on its ability to subvert host functions by escaping phagocytic vacuoles and spreading from cell to cell without exiting the intracellular milieu. In the last two decades, multiple studies have aimed at identifying virulence factors and deciphering the mechanisms by which *L. monocytogenes* survives in a wide range of environments (9, 13).

One of the most valuable genetic tools used to study bacteria is the transposon. Transposons can be used to perform high-throughput mutagenesis of an entire chromosome, generating banks of mutants that can be screened for identification of factors related to specific bacterial functions. The transposon delivery systems that are currently available for use with *L. monocytogenes* are not ideal for these types of studies (4, 7, 14). For example, the most commonly used transposon delivery system, Tn*917*-LTV3, is more than 22 kb in size and has a low efficiency of transposition and a high rate of vector retention (4). In this study, we aimed at designing a transposon delivery system that is more suitable to high-throughput mutagenesis.

In recent years, *Himar1 mariner* has been used as the transposon of choice in performing high-throughput mutagenesis in many different bacterial species, including low-GC-content gram-positive species (1, 2, 18). *Himar1* was originally isolated from the horn fly, *Haematobia irritans*, and is a member of the *Tc1*/*mariner* superfamily of transposable elements (21). The *Tc1*/*mariners* are the most-widespread transposons in nature. These elements require no factors for transposition other than their self-encoded transposases (16), a feature that makes them ideal candidates for development into generalized genetic tools. Moreover, the *mariner* requirement for insertion is the dinucleotide TA, which makes it perfect for transposition into low-GC-content organisms such as *L. monocytogenes* (39% GC). We reasoned that a *mariner*-based transposition

TABLE 1. Bacterial strains and vectors used in this study

Strain or vector	Genotype or relevant feature	Reference or source
10403S	*L. monocytogenes* serotype 1/2a	3
CU1065	*B. subtilis* W168 *trpC2 attSP*β	J. D. Helmann
DH5α	*E. coli supE44* Δ*lacU169* (ϕ80*lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Lab stock
pCR2.1-TOPO	Cloning vector from Invitrogen	
pDG780	Contains a gram-positive *kan* resistance cassette	12
pKSV7	Shuttle vector with gram-positive pE194ts *ori*	22
pLTV3	Tn*917* delivery vector	4
pMC1	Gram-positive *cat* gene from pPL2 cloned into pCR2.1-TOPO	This study
pMC3	Gram-positive *cat* gene from pMC1 cloned into pMMOrf	This study
pMC14	Gram-positive *kan* gene from pDG780 cloned into pPL2	This study
pMC25	*mariner* delivery vector with *L. monocytogenes* promoter P_{P60}	This study
pMC30	*mariner* delivery vector with *L. monocytogenes* promoter P_{actA}	This study
pMC38	*mariner* delivery vector with *B. subtilis* promoter P_{mrgA}	This study
pMC39	*mariner* delivery vector with *B. subtilis* promoter P_{katA}	This study
pMMOrf	Contains 5′ and 3′ ITR from *Himar1*	15
pNF1100	Contains a copy of *Himar1* derived from pMEnt-*neo*	N. E. Freitag; 16
pPL2	Site-specific shuttle integration vector	17
pPL3e	pPL2 derivative with gram-positive *ermC* gene	10

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TABLE 2. Oligonucleotide primers used in this study

No. of primer	Sequence 5′→3′[a]	Characteristic (reference)
155	TTGGATCCCGGAGACGGTCACA	BamHI
156	CGCATCTGTGCGGTATTTCA	
188	ATCCGCATGCTGCAAGGCGATTAAGT	SphI
194	CGGGTACCATCACACGCAAAAAGGA	KpnI
195	CGGGTACCTAAATTCAAAATCTATC	KpnI
205	GGTATAGCATATGAATCGCATCCGATTGCAG	NdeI
206	TGTCAGACATATGGGCACACGAAAAACAAGT	NdeI
207	GGCCACGCGTCGACTAGTACNNNNNNNNNNG TAAT	ARB1B (8)
208	GGCCACGCGTCGACTAGTAC	ARB2 (8)
234	GCGGATCCAGA*GGAG*TTTTATGAATATGGAA AAAAAGGAATTTCGTGTTT	BamHI and RBS
247	GCGGTACCTATCATCAATACTATA	KpnI
248	CAGGATCCGTGATCTGTTGACTTAAT	BamHI
249	GCGGTACCTTTTCTTTGATGCTGA	KpnI
250	GCGGATCCAATTTATAAGAACATAAT	BamHI
254	CGTGGAATACGGGTTTGCTAAAAG	
255	CAGTACAATCTGCTCTGATGCCGCATAGTT	
256	TAGTTAAGCCAGCCCCGACACCCGCCAACA	
257	CTTACAGACAAGCTGTGACCGTCT	
269	GCTCTGATAAATATGAACATGATGAGTGAT	
270	TGTGAAATACCGCACAGATGCGAAGGGCGA	
271	GGGAATCATTTGAAGGTTGGTACT	

[a] Restriction sites are underlined, and the ribosome binding site is italicized.

system would be an excellent tool for the entire community of scientists working with *L. monocytogenes*.

Construction of *mariner*-based transposon delivery vectors. The plasmids and primers used for construction of a *mariner* delivery vector for *L. monocytogenes* are listed in Tables 1 and 2, respectively. pPL2 (17) and pDG780 (12) were digested with SacI and XhoI, and the gram-positive kanamycin (*kan*) resistance cassette from pDG780 was ligated into pPL2 to create pMC14. The gram-positive chloramphenicol acetyltransferase gene (*cat*) from pPL2 was amplified by PCR with primer pair Marq155/156 and ligated into pCR2.1-Topo (Invitrogen) to create pMC1. pMC1 was digested with BamHI and ligated into the BglII site of pMMOrf (15) between the 5′ and 3′ *Himar1*-inverted terminal repeats (ITR), creating pMC3. The *Himar1* transposase gene (*tpase*) (16) was amplified by PCR from pNF1100 (provided by Nancy Freitag) with primer pair Marq188/234. The promoter regions of the *Bacillus subtilis* CU1065 (provided by John Helmann) *mrgA* and *katA* genes were amplified by PCR, using primer pairs Marq247/248 and Marq249/250, respectively. P_{mrgA}, P_{katA}, and *tpase* PCR fragments were digested with BamHI, and each promoter was individually ligated to *tpase*. The P_{mrgA}-*tpase* and P_{katA}-*tpase* ligation products were amplified by PCR with primer pairs Marq247/188 and Marq249/188, respectively. The P_{mrgA}-*tpase* and P_{katA}-*tpase* PCR products and pMC14 were digested with KpnI and SphI. The pMC14 fragment comprising P4*oriT*, p15A*ori*, gram-negative *cat*, and gram-positive *kan* genes was ligated with P_{mrgA}-*tpase* and P_{katA}-*tpase*, respectively. The ITR-*cat*-ITR fragment from pMC3 was ligated at the KpnI and XhoI sites, and the gram-positive *cat* gene was later replaced by *ermC*. This replacement was done by amplifying *ermC* from pPL3e (10) with primer pair Marq205/206, digesting the PCR products and vector with NdeI, and replacing the *cat* gene between the ITR with *ermC*. Last, the temperature-sensitive origin of replication pE194ts *ori* in pKSV7 (22) was amplified by PCR, using primer pair Marq194/195, digested with KpnI, and ligated into each vector, creating pMC38 (P_{mrgA}) and pMC39 (P_{katA}) (Fig. 1). The vectors are 8,172 bp (pMC38) and 8,297 bp (pMC39), and the transposon itself is 1,395 bp.

The *Himar1* transposase and the ITR sequences are the only two factors required for transposition; thus, successful expression of the transposase in *L. monocytogenes* is the key element. In our initial constructs, the *L. monocytogenes* p60 (pMC25) and *actA* (pMC30) promoters were used to direct transcription of the transposase gene. This approach failed, as the entire plasmid invariably integrated into the chromosome. However, when we used *B. subtilis* to evaluate the efficiency of transposition with these same vectors, we obtained a very low level of plasmid retention (data not shown). We reasoned that the *Listeria* species promoters were responsible for mediating plasmid integration into the chromosome of *L. monocytogenes* and sought to use two *B. subtilis* σ^A-dependent promoters (P_{mrgA} and P_{katA}) with no sequence similarity to the *Listeria* genome (6). This approach was very successful.

Evaluation of the *mariner*-based transposon delivery vector. pMC38 and pMC39 were preferentially transferred into *L. monocytogenes* strain 10403S by electroporation, as the efficacy of transfer by conjugation was very low. Transformants were selected at 30°C on brain heart infusion (BHI) plates supple-

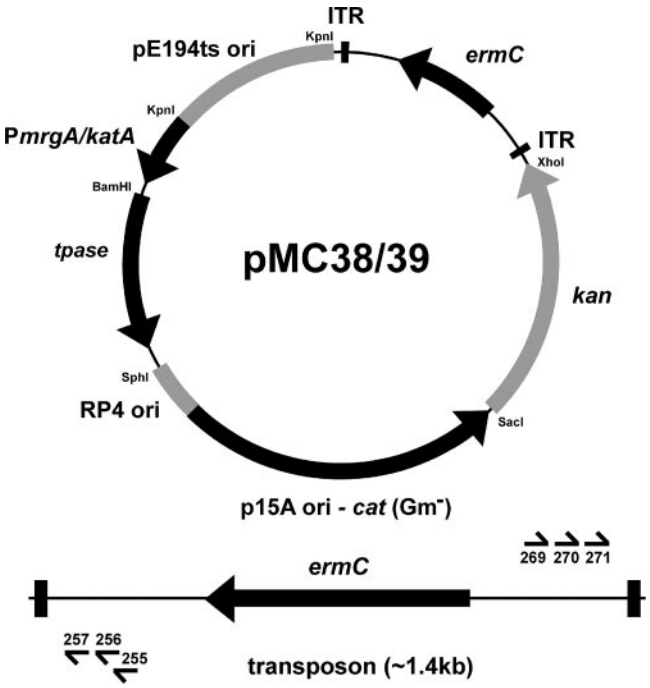

FIG. 1. Physical map of the *mariner*-based transposon delivery vectors pMC38 and pMC39. The vectors comprise the *Escherichia coli* p15A low-copy-number replication origin (5), the RP4 *ori* for conjugative transfer (20), the pE194ts gram-positive temperature-sensitive replication origin (11, 25), a gram-negative chloramphenicol resistance gene (*cat*) (5), a gram-positive noninducible erythromycin resistance gene (*ermC*) (25) flanked by the 29-bp ITR of the *Himar1 mariner* (15), a gram-positive kanamycin resistance gene (*kan*) (12) as a screening marker for loss of the plasmid, and the *Himar1 mariner* transposase gene (*tpase*) (16). The indicated restriction sites represent those used for cloning, but they are not necessarily unique. Gm^-, gram negative.

TABLE 3. Comparison of transposon delivery vectors

Delivery vector	Size of vector	Size of transposon (bp)	Total no. of mutants (CFU/ml ± SD)[a]	% ± SD of CFUs bearing vector
pLTV3(4)	22.1 kb	14,861	$(9.80 \pm 2.00) \times 10^4$	>50[b]
pMC38	8,172 bp	1,395	$(1.73 \pm 1.07) \times 10^6$	2.4 ± 1.7
pMC39	8,291 bp	1,395	$(1.08 \pm 0.76) \times 10^6$	0.85 ± 0.01

[a] The total number of mutants generated by each vector was obtained from the results of three independent libraries for pLTV3 and pMC38 and two independent libraries for pMC39. SD, standard deviation.
[b] Estimated number.

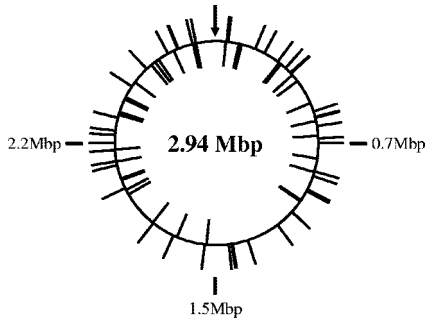

FIG. 2. Locations of 77 sequenced *Himar1* insertions in the chromosome of *L. monocytogenes*. The bars located outside the circle indicate transposons that were oriented in the same direction as the positive strand, whereas the bars located inside the circle indicate transposons that were oriented in the opposite direction. Each quarter of the chromosome is identified with the approximate base pair, except for bp 1, which is identified by an arrow.

mented with erythromycin at 5 μg/ml. Individual colonies were grown overnight in BHI with erythromycin and kanamycin (10 μg/ml) at 30°C with shaking. The cultures were diluted 1/200 in broth with erythromycin, grown for 1 h at 30°C with shaking, and then shifted to 40°C for about 6 h until the optical density at 600 nm was between 0.3 and 0.5. Aliquots of the culture were plated on BHI agar supplemented with erythromycin and incubated at 40°C. Individual colonies were picked and plated in parallel on BHI agar supplemented with either erythromycin or kanamycin to evaluate the rate of plasmid retention. The rate of plasmid retention was calculated by dividing the number of kanamycin-resistant colonies (due to plasmid retention) by the number of erythromycin-resistant colonies (total number of mutants with the plasmid or with the transposon only). The same procedures were used to evaluate the Tn*917*-based transposition system, Tn*917*-LTV3 (4), except that 1 μg/ml erythromycin plus 25 μg/ml lincomycin were used for selection of transposon mutants and 12.5 μg/ml tetracycline was used to evaluate plasmid retention.

In general, we obtained 10-fold-more mutants with the *mariner*-based vectors than with the Tn*917*-based vector (Table 3). Plasmid retention was less than 2.5% for the *mariner*-based vectors, whereas it was more than 50% with the Tn*917*-based vector. Therefore, by comparing the numbers of transposon insertion mutants (colonies that have lost the plasmid) generated by the two systems, we estimated that the efficiency of transposition of the *mariner*-based vectors was more than 20-fold higher than that of the Tn*917*-based vector.

To evaluate the randomness of transposition, we arbitrarily picked 100 erythromycin-resistant colonies from one library, performed Southern blot analysis, and identified the sites of transposon insertion. For Southern blot analysis, a 400-bp fragment within the *ermC* gene was amplified from pMC38 by PCR, using primer pair Marq206/254. The probe was labeled with alkaline phosphatase, using the AlkaPhos Direct labeling system from Amersham Biosciences. Of the 100 mutants, 84 had a single transposon insertion, whereas 16 had two-to-three insertions (data not shown). To identify the sites of transposon insertion, we initially performed arbitrary PCR to amplify the DNA sequences flanking the transposon (8). DNA was amplified from either end of the transposon with a series of two rounds of PCR with *Taq* polymerase in the first round and Expand High Fidelity polymerase (Roche) in the second round. In each round, a transposon-specific primer and an arbitrary primer were used. The arbitrary primers Marq207 and Marq208 were previously identified as ARB1B and ARB2 by Garsin et al. (8). The approximate locations of transposon-specific primers are illustrated in Fig. 1. In the first round, DNA fragments from the left and right ends of the transposon were amplified with primer pairs Marq207/255 and Marq207/269, respectively. For the second round, 5 μl of a 1/25 dilution from the first round of PCR was used in a 20-μl reaction. DNA fragments from the left and right ends of the transposon were amplified with primer pairs Marq208/256 and Marq208/270, respectively. The PCR products were sequenced, using primers Marq257 and Marq271 for the left and right ends of the transposon, respectively. The Biotechnology Research Center of Cornell University performed the sequencing with an Applied Biosystems automated 3730 DNA analyzer. The ListiList website (http://genolist.pasteur.fr/ListiList/) was used for sequence analysis.

Readable sequencing results were obtained from 77 transposon insertion mutants, including one pair of siblings and a series of three genes that were hit twice. For the 77 mutants, 36 insertions were in positive-strand open reading frames, 29 in negative-strand open reading frames, and 12 in intergenic regions (Fig. 2). The mutants distributed evenly along the *L. monocytogenes* genome, and there was no bias in terms of the transposon orientation. We further aligned all the insertion sites, and no sequence specificity beyond the known requirement of the dinucleotide TA was found (data not shown).

Taken together, these results show that the newly designed *mariner* delivery vectors are powerful genetic tools to study *L. monocytogenes*. The *mariner*-based system outachieves the Tn*917*-based system in the following aspects: transposition efficiency, randomness, and a low rate of plasmid retention. The temperature-sensitive replicon (pE194ts *ori*) and the noninducible erythromycin resistance gene (*ermC*) are common to many gram-positive bacteria. We believe that this *mariner*-based system will be a great tool for the entire community of scientists working with *L. monocytogenes* and other low-GC gram-positive bacteria.

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