Insertional Mutagenesis of *Listeria monocytogenes* with a Novel Tn917 Derivative That Allows Direct Cloning of DNA Flanking Transposon Insertions

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To carry out efficient insertional mutagenesis in *Listeria monocytogenes* and to facilitate the characterization of disrupted genes, two novel derivatives of Tn917 were constructed, Tn917-LTV1 and Tn917-LTV3. The derivatives (i) transpose at a significantly elevated frequency, (ii) generate transcriptional *lacZ* fusions when inserted into a chromosomal gene in the appropriate orientation, and (iii) allow the rapid cloning in *Escherichia coli* of chromosomal DNA flanking transposon insertions. The rapid cloning of DNA flanking insertions is possible because the transposon derivatives carry CoIE1 replication functions, a cluster of polynucleotide cloning sites, and antibiotic resistance genes selectable in *E. coli* (*bla* in the case of Tn917-LTV1; *neo* and *ble* in the case of Tn917-LTV3). The enhanced transposition frequency of Tn917-LTV1 and Tn917-LTV3 (about 100-fold in *Bacillus subtilis*) is believed to be due to the fortuitous placement of vector-derived promoters upstream from the Tn917 transposase gene. In *L. monocytogenes*, Tn917-LTV3 transposed at a frequency of $8 \times 10^{-4}$ when introduced on a pE194Ts-derived vector and generated at least eight different auxotrophic mutations. Two nonhemolytic insertion mutants of *L. monocytogenes* were isolated, and DNA flanking the transposon insertions was cloned directly into *E. coli*, making use of the CoIE1 rep functions and *neo* gene carried by Tn917-LTV3. Both insertions were shown to be within *hlyA*, the *L. monocytogenes* hemolysin structural gene. Although Tn917-LTV1 and Tn917-LTV3 were constructed specifically for genetic analysis of *L. monocytogenes*, their enhanced transposition frequency and convenience for cloning of DNA adjacent to sites of insertions make them the transposon derivatives of choice for insertional mutagenesis in any gram-positive bacteria that support replication of pE194Ts.

*Listeria monocytogenes* is a gram-positive, facultative intracellular pathogen responsible for infrequent but often severe infections in humans characterized by meningitis, meningocerebralitis, septicaemia, and fetal death (37). *L. monocytogenes* is ubiquitous in nature and, in addition, can be isolated from a wide variety of warm-blooded animals (36). Historically, *L. monocytogenes* has been used as a model intracellular pathogen for studies of cell-mediated immunity (18, 25, 30). Recently, it has been shown that *L. monocytogenes* can infect and grow within a wide variety of cultured animal cells (16, 23, 33) and spread cell-to-cell without ever leaving the host cytoplasm (39). Very little is understood, however, about bacterial determinants necessary for cell attachment, uptake, intracellular growth, or cell-to-cell spread during the course of an infection. Such studies have been hindered by the lack of tools for genetic manipulation of *L. monocytogenes.*

Transposon-mediated insertional mutagenesis was recently demonstrated in *L. monocytogenes* by the introduction of the conjugative transposons TnI545 (11) and Tn916 (15). TnI545 was delivered into *L. monocytogenes* at a frequency of approximately $10^{-8}$ through conjugation with strains of *L. monocytogenes* harboring this transposon (17), and Tn916 was introduced through conjugation with Tn916-containing strains of *Enterococcus faecalis* at a frequency of $10^{-6}$ (20). These relatively low frequencies of transposition make it inconvenient to carry out large-scale mutagenesis, however, and the randomness of insertion of conjugative transposons is limited by the requirement for sequence homology between both ends of the elements and sequences surrounding the sites of integration (9, 35). Cossart et al. (10) have recently introduced the Tn3-like transposon Tn917 into *L. monocytogenes*, carried on vector pTV1, and demonstrated its utility for insertional mutagenesis. This transposon exhibits a high degree of insertional randomness in *Bacillus subtilis* as well as many other gram-positive bacteria and generates extremely stable insertional mutations (47, 49). More importantly, extensive information exists concerning the physical and genetic organization of Tn917, which facilitates altering the transposon in ways that might enhance its utility in species such as *L. monocytogenes* (38, 48).

To take full advantage of the fact that Tn917 can function in *L. monocytogenes*, we have constructed two modified forms of the transposon, Tn917-LTV1 and Tn917-LTV3. These derivatives were designed to include the following features. First, they are carried by highly temperature-sensitive derivatives of vector pE194Ts (41). This simplifies the recovery of chromosomal insertions. Second, they contain a promotorless copy of the *Escherichia coli lacZ* gene oriented such that insertions into chromosomal genes can generate transcriptional *lacZ* fusions. Third, they contain, immediately downstream from the *lacZ* coding sequence, an *E. coli* cloning vector that includes a gene selectable in *E. coli*, a gene selectable in *B. subtilis*, CoIE1 replication functions, an M13 origin of replication, and a cluster of polynucleotide cloning sites. The polynucleotide sites facilitate the recovery in *E. coli* of chromosomal DNA adjacent to sites of insertion, particularly DNA on the promoter-proximal side of transposon-mediated *lacZ* fusions. An extremely impor-

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tant but unanticipated advantage of these transposon derivatives is the fact that they exhibit a much higher frequency of transposition than previously described versions of Tn917 (as much as 100-fold greater in _B. subtilis_). This should greatly simplify obtaining libraries of transposon-mediated _lac_ fusions in a wide range of bacteria.

To investigate the randomness of Tn917 insertions in _L. monocytogenes_ and to test the utility of our modified derivatives of the transposon, several independent insertion libraries were obtained with Tn917-LTV3 and screened for various kinds of insertional mutations. Insertional auxotrophic mutations in at least eight distinct loci were obtained. The results suggest that hotspots may exist in the _L. monocytogenes_ chromosome where Tn917 insertions are more frequent but that the overall degree of randomness is of a high order. Several insertions were also obtained within the _hlyA_ gene, which encodes a hemolysin protein whose activity is a critical virulence determinant of the organism. A physical analysis of these _hlyA::Tn917-LTV3_ insertions revealed them to be distributed throughout the _hlyA_ coding sequence and in both orientations with respect to the transcriptional polarity of the gene. Insertions in the appropriate orientation expressed β-galactosidase at high levels. Two of the insertions were used to rescue, into _E. coli_, chromosomal DNA flanking the insertion junctions.

**MATERIALS AND METHODS**

**Bacterial strains.** The _B. subtilis_ host for plasmid constructions and transposition studies was BD170 ( _trpC2 thr-5_ ) (13). The _E. coli_ host for plasmid constructions was HB101 [ _hsdS20 (r-h, m-h_) recA13 ara-14 proA2 lacY1 galK2 rpsL20(Sm') xyl-5 ml-1 supE44 F- λ- ] (5). The _E. coli_ host for recovery of _L. monocytogenes_ DNA sequences flanking transposon insertions was MC1061 [ _hsdR recB araD139 Δ araABC-lev7679 Δ lacX74 galU galK rpsL thi_ ] (7). The _L. monocytogenes_ host for transposition studies was 10403S (2).

**Culture media and reagents.** Unless otherwise specified, all strains were cultured on Luria-Bertani (LB) medium (12). All antibiotics were purchased from Sigma and were used at the following concentrations: 12.5 μg of tetracycline per ml, 1 μg of erythromycin per ml, 25 μg of lincomycin per ml, 10 μg of chloramphenicol per ml, 50 μg of ampicillin per ml, and 20 μg of kanamycin per ml. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was used at 40 μg/ml in agar media. β-Galactosidase activity in LB liquid cultures was assayed as described by Miller (28). All restriction enzymes and DNA-modifying enzymes were used according to the specifications of the manufacturer.

**Construction of pLTV1 and pLTV3.** The extreme replication thermosensitivity of pE194Ts is due to a point mutation in its _repC_ gene (41). This mutation was transferred to pVT51 (50) by transforming _B. subtilis_ PY339 containing pBD95Ts (46) to _Tc_ with pVT51 DNA linearized by digestion with _PstI_. Among the in vivo products of recombination was pTV51Ts, whose temperature-sensitive replication properties were evaluated on media containing erythromycin and lincomycin. To obtain pLTV1 (Fig. 1), equimolar quantities of _HamIII_-digested pTV51Ts and _HindIII_-digested pBG5 DNA (50) were ligated at a total DNA concentration of 10 μg/ml after treating digested fragments with the Klenow fragment of DNA polymerase I to produce flush ends (43), and the ligation products were used to transform _E. coli_ HB101 to _Tc_ by using standard methods (34). To obtain pLTV3, the _bla_ gene in pBG5 was replaced with the _neo_ and _ble_ genes from _Tn5_ (26) prior to insertion into Tn917-lac. This was accomplished by insertion of the 1.9-kilobase HindIII-BamHI fragment from _Tn5_ containing its _neo_ and _ble_ genes into the 4.5-kilobase _PvuI-EcoRI_ backbone of pBG5, after treatment of fragments to produce flush ends (43). To recover pBG5-neo, the ligation mixture was used to transform _E. coli_ HB101 and Km' transformants containing pBG5-neo were selected. Plasmid pBG5-neo was digested with _HindIII_, treated with Klenow fragment, and ligated in the presence of an equimolar amount of _Smal_-digested pTV51Ts. To recover pLTV3, the ligation mixture was used to transform _E. coli_ HB101, with selection for _Tc_ transformants. Plasmid DNA was prepared from _E. coli_ HB101 transformants harboring pLTV1 and pLTV3 after growth in LB (12) containing ampicillin (pLTV1) or kanamycin (pLTV3).

Plasmids pLTV1 and pLTV3 were introduced into _B. subtilis_ BD170 by transformation of naturally competent cells (1). _B. subtilis_ transformants containing pLTV1 and pLTV3 were designated strains DP-B982 and DP-B983, respectively. Plasmid pLTV3 was introduced into _L. monocytogenes_ 10403S by transformation of protoplasts as described below.

**Transformation of _L. monocytogenes_.** Protoplasts of _L. monocytogenes_ were prepared and transformed with plasmid DNA by using a modification of previously described procedures (42). Log-phase _L. monocytogenes_ cells at an optical density (600 nm) of 0.8 were harvested from a 20-ml brain heart infusion culture and were washed in 20 ml of H₂O. The cells were suspended in 2 ml of 0.1 M sodium phosphate (pH 7.0), and 0.5-ml portions were dispensed into four sterile 15-ml Corex tubes. To each tube, 4.5 ml of a solution containing 0.67 M sodium phosphate (pH 7.0) and 0.34 mg of bile salts (Sigma) per ml was then added. A 1 mg/ml solution of porcine pancreas lipase (Sigma) in 0.1 M sodium phosphate (pH 7.0) was centrifuged at 10,000 × _g_ in a microcentrifuge for 30 s, and 1, 3, 5, or 10 μl of the clear supernatant was added to each tube of cells. The cells were incubated at 37°C with gentle shaking for 15 min, 50 μl of 0.1 M CaCl₂ was added to each tube, and the incubation was continued for 45 min with gentle shaking. A 3-ml portion of 0.155 M NaCl was added to each tube, and the cells were pelleted at 4,300 × _g_ for 15 min. Each cell pellet was washed once with 5 ml of 0.155 M NaCl and suspended in 0.5 ml of 0.155 M NaCl by vortexing vigorously. To each tube, 4.4 ml of a solution containing 0.34 mg of lysozyme per ml, 0.03 M Tris hydrochloride (pH 6.7), and 0.45 M sucrose was then added. The cells were incubated at 37°C for 15 min with gentle shaking, 0.1 ml of 1 M MgCl₂ was added to each tube, and the incubation was continued for 3 h at 37°C with gentle shaking. Formation of protoplasts was followed by phase-contrast microscopy. Cells from the tube showing the highest percentage of protoplasts were pelleted at 7,600 × _g_ at 25°C, washed once in 5 ml of SMMP (2 × Difco antibiotic medium no. 3, 0.5 M sucrose, 0.02 M maleate, 0.02 M MgCl₂ [pH 6.5], adjusted with NaOH), and suspended in 200 μl of SMMP. For transformation, 1 μg of pLTV3 DNA was added and the solution was mixed by gentle swirling followed by the addition of 600 μl of 40% PEG (Sigma; _M_w_ 2,500) in 2 × SMMP (SMMP is SMMP without Difco medium). The solution was gently mixed by swirling, and after 3 min at room temperature, 6 ml of SMMP was added. The protoplasts were pelleted at 7,600 × _g_ for 15 min at 25°C, resuspended in 200 μl of SMMP containing a subinhibitory but inducing concentration of erythromycin (0.04 μg/ml), and incubated at 30°C for 1 h to allow the inducible expression of the.
transposon-encoded *erm* gene. Protoplasts were plated onto DM3 plates (8) (0.5 M sodium succinate [pH 7], 0.5% Casamino Acids, 0.5% yeast extract, 0.35% KHPO₄, 0.15% KH₂PO₄, 0.5% glucose, 0.02 M MgCl₂, 0.01% bovine serum albumin, 0.8% agar) containing 1 μg of erythromycin per ml and incubated at 30°C. Erythromycin-resistant transformants containing pLTV3 formed small L-form colonies (22) after 2 days and large colonies, in which the bacterial cell walls had regenerated, after 3 to 4 days. A *L. monocytogenes* transformant containing pLTV3 was designated strain DP-L910. All unspecified reagents used were from Sigma.

**Determination of transposition frequencies.** Single colonies of *B. subtilis* DP-B982(pLTV1), DP-B983(pLTV3), or *L. monocytogenes* DP-L910(pLTV3) were picked after overnight growth at 30°C on LB agar containing erythromycin, lincomycin, and tetracycline and used to inoculate 10 ml of LB cultures containing the above antibiotics. *B. subtilis* PY313 containing pTV1Ts (46) was also grown overnight and used to inoculate a culture as above, except that chloramphenicol was used in place of tetracycline. All four cultures were grown with aeration at 30°C to an optical density (600 nm) of approximately 0.4. Dilutions of each culture were made, and the number of CFU at the permissive (30°C) and nonpermissive (47°C for *B. subtilis* and 41°C for *L. monocytogenes*) temperatures for plasmid replication were determined on LB agar containing erythromycin and lincomycin.

The transposition frequencies were calculated by dividing the titers at the nonpermissive temperatures by those at 30°C. Transposition frequency determinations were performed in triplicate for each strain, and the frequencies were then averaged.

**Isolation of transposon insertions.** A single colony of *L. monocytogenes* DP-L910(pLTV3) was used to inoculate 2 ml of brain heart infusion containing erythromycin, lincomycin, and tetracycline, and the culture was grown overnight at 30°C to stationary phase. The overnight culture was inoculated 1/800 into brain heart infusion containing erythromycin and lincomycin, and the bacteria containing chromosomal transposon insertions were selected for by growth with aeration at 41°C until stationary phase. This treatment resulted in a population of cells of which 90% were Em⁺, Lm⁺, and Tc⁺, indicating loss of pLTV3 with retention of transposon insertions into the chromosome. Aliquots of the culture were directly frozen in LB broth at −70°C until later use. The frozen aliquots from a single culture will be referred to as transposon insertion libraries of *L. monocytogenes*.

**Characterization of insertional mutants.** A total of 1,000 colonies of *L. monocytogenes* from each of 10 separate transposon insertion libraries were patched onto minimal medium (44) to screen for 18 common auxotrophic types. Mutant strains unable to grow on the minimal media were subsequently analyzed to determine their specific auxo-
trophic requirements, as described by Davis et al. (12). Nonhemolytic transposon insertion mutants of *L. monocytogenes* were isolated by plating insertion libraries directly onto blood agar and screening for colonies lacking a zone of hemolysis.

**Cloning of DNA flanking transposon insertions.** Chromosomal DNA from transposon insertion mutants of *L. monocytogenes* was isolated as described by Flamm et al. (14). Chromosomal DNA was digested with *XbaI*, followed by ligation in a 100-μl volume at a DNA concentration of 5 μg/ml. The ligated DNA was concentrated by ethanol precipitation and used to transform *E. coli* HB101 to Km’. Plasmid DNA was isolated from Km’ transformants and analyzed by restriction enzyme analysis.

**Southern blot analysis.** Southern blot analysis of transposon insertions in the *L. monocytogenes* hemolysin structural gene, *hlyA* (27), was done as previously described (6).

**RESULTS**

**Construction of pLTV1 and pLTV3.** In previous work (48), it was shown that insertion of foreign DNA into a particular region of Tn917 near the *erm*-proximal end did not affect transposition. Making use of two unique restriction sites present within this region of the transposon derivative in pTV51Ts, linearized pBG5 and pBG5-neo were inserted to create pLTV1 and pLTV3, respectively (Fig. 1). The Tn917-derivatives in pLTV1 and pLTV3 were designated Tn917-LTV1 and Tn917-LTV3, respectively. The structures and functional properties of pLTV1 and pLTV3 were confirmed by the following criteria: restriction enzyme analysis (data not shown); ability to confer resistance to tetracycline and ampicillin (pLTV1) or tetracycline and kanamycin (pLTV3) in *E. coli*; ability to confer resistance to erythromycin, lincomycin, tetracycline, and chloramphenicol in *B. subtilis* and *L. monocytogenes*; and ability of *B. subtilis* strains containing pLTV1 or pLTV3 to form blue colonies on LB agar containing 40 μg of X-gal per ml (29). The parental *L. monocytogenes* 10403S had a low endogenous β-galactosidase activity, forming very light blue colonies on LB agar after 48 h of growth at 30°C. However, many transposon insertions into transcriptionally active regions of the *L. monocytogenes* chromosome were easily detected above this background, causing colonies containing them to turn dark blue on X-gal plates. When subsequently examined in liquid culture, some of these insertions were found to produce higher than 1,000-fold more β-galactosidase activity than the wild type.

To determine whether the insertion of pBG5 or pBG5-neo into Tn917-lac affected transposition, the transposition frequencies of Tn917-LTV1 and Tn917-LTV3 in *B. subtilis* were determined and compared with an unaltered version of Tn917 (Table 1). The transposition frequency of Tn917 in *B. subtilis* was 5.8 × 10^-5, which was similar to that previously reported (47). Surprisingly, the transposition frequency of Tn917-LTV1 and Tn917-LTV3 in *B. subtilis* was approximately 100-fold greater. Transposition of Tn917-LTV3 in *L. monocytogenes* was approximately sevenfold less frequent than in *B. subtilis*, which was similar to that previously reported for Tn917 (10).

**Randomness of transposon insertion in *L. monocytogenes.*** To evaluate the randomness of Tn917 insertions in the *L. monocytogenes* chromosome, 10,000 insertions (1,000 from each of 10 independent libraries) were characterized to determine whether they included different kinds of auxotrophic mutations. Insertional auxotrophic mutants were isolated at a frequency of 0.82%. Among the 82 auxotrophs isolated, the following common requirements were found: 48 adenosine, 14 uracil, 8 proline, 5 glycine, 3 nicotinic acid, 2 phenylalanine, 1 glutamine, and 1 aromatic amino acids (Table 2). Wild-type *L. monocytogenes* strains require cysteine, glutamine, isoleucine, valine, arginine, histidine, methionine, tryptophan, thioctic acid, riboflavin, thiamine, and biotin for growth on a synthetic minimal medium, which would have prevented the detection of several common auxotrophic types (44). Thus, we obtained 8 of 18 possible common auxotrophic types screened for, suggesting that Tn917-LTV3 can insert into the *L. monocytogenes* chromosome with a relatively high degree of randomness. Nevertheless, as in *B. subtilis*, there would appear to be hotspot regions of the chromosome where insertions are more abundant (49). At least one of these hotspot regions apparently contains genes required for adenine biosynthesis.

To evaluate the insertional randomness within a single chromosomal locus in *L. monocytogenes*, we isolated insertions in the listeriolysin O structural gene, *hlyA* (27), that caused a nonhemolytic phenotype. Nonhemolytic insertion mutants were detected at a frequency of 6 × 10^-3. Eight independent insertions were mapped within *hlyA*, of which three formed blue colonies on LB agar containing X-gal and had β-galactosidase activities in solution 1,000-fold higher than the wild type. As hemolysin is strongly expressed on LB agar (A. Camilli and D. Portnoy, unpublished data), this suggested that transcriptional fusions between *hlyA* and the transposon-containing *lacZ* gene had formed in these three mutants. The approximate sites of insertion and the transposon orientations of the eight *hlyA* insertions are shown in Fig. 2.
from the transposon insertion directly above its 3' end. Flanking DNA from two hlyA insertions are shown below.

**Tn917-LTV1**

Using the rapid cloning of flanking DNA. Unexpectedly, the transcriptional orientations of the transposon-associated *lacZ* gene are indicated by horizontal arrows below each insertion. The locations of cloned flanking sequences (insert sequences A and B) from two transposon insertions are shown below *hlyA*. Insert A was obtained, after digestion with *XbaI*, from the transposon insertion directly above the 5' end of insert A. Insert B was similarly obtained from the transposon insertion directly above its 3' end. Mapping was based on single and double digestions of the cloned flanking sequences with the appropriate restriction enzymes (data not shown), on Southern blot analysis of wild-type *L. monocytogenes* chromosomal DNA digests probed with labeled inserts A and B (data not shown), and on the published *hlyA* sequence (27).

**Direct cloning of DNA flanking transposon insertions.** To demonstrate that chromosomal DNA flanking the sites of transposon insertions could be cloned directly into *E. coli* by using the CoIE1 replicon within Tn917-LTV3, we cloned flanking DNA from two *hlyA* insertions. Taking advantage of known restriction sites in the vicinity of *hlyA*, DNA flanking the insertions was easily cloned by using the restriction enzyme *XbaI*. Because an *XbaI* site is present in the polylinker of Tn917-LTV3, sequences cloned by using *XbaI* digests extended in one direction only, from the site of insertion (Fig. 2 and 3).

**DISCUSSION**

To facilitate the study of bacterial determinants of *Listeria monocytogenes* pathogenicity, we have constructed transposition-proficient derivatives of Tn917-lac containing CoIE1 replication functions. By using one of these derivatives (Tn917-LTV3), we have shown that Tn917 can insert into the *L. monocytogenes* chromosome with a relatively high degree of randomness, generating *lacZ* transpositional fusions when insertions occur within genes in the appropriate orientation. The presence of CoIE1 replication functions and polylinker cloning sites allowed the convenient and rapid cloning of flanking DNA. Unexpectedly, both derivatives, Tn917-LTV1 and Tn917-LTV3, exhibited enhanced transposition frequencies in *B. subtilis*. Although the reason for the increased transposition frequencies was not determined, it is possible that the level of expression of the Tn917 transposase is increased in these constructs due to transcriptional readthrough from within the inserted CoIE1 replicons.

In the case Tn917-LTV1, the *lac* promoter adjacent to the polylinker cluster in pBG5 (50) is positioned appropriately to direct transcription toward the Tn917 transposase gene. This promoter is deleted in Tn917-LTV3 but is replaced by the promoter for the Tn5 Smr' gene (26). The increased frequencies of transposition of pLTV1 and pLTV3 should greatly facilitate their use for insertional mutagenesis in *B. subtilis*, and potentially in other gram-positive bacteria as well, by reducing the culture volumes necessary to produce transposon insertion libraries.

Transposons Tn917-LTV1 and Tn917-LTV3 differ only in the gram-negative antibiotic resistance marker contained within their CoIE1-derived sequences. Although we would expect that the gram-negative *bla* gene present in Tn917-LTV1 would not confer resistance to ampicillin in *L. monocytogenes*, we considered it undesirable to introduce this gene into a pathogen for which β-lactam antibiotics are clinically important therapeutics. Thus, we replaced the *bla* gene with the *neo* and *ble* genes from Tn5 in the construction of Tn917-LTV3. Although Tn917-LTV3 might be the transposon of choice for other gram-positive pathogens as well (*e.g.*, *Streptococcus* spp.), Tn917-LTV1 should prove generally more useful for insertional mutagenesis in nonpathogenic gram-positive species since it contains additional unique restriction sites within its polylinker region. Schematic examples of *lacZ* fusions mediated by Tn917-LTV1 and Tn917-LTV3 are shown in Fig. 3 to illustrate the kinds of restriction sites available in both transposon derivatives for the cloning of DNA flanking transposon insertions.

The randomness of Tn917 insertions into chromosomal targets has been most extensively investigated in *B. subtilis* (40) and *B. megaterium* (3), although the transposon has been shown to function efficiently in a broad range of bacteria, including both gram-positive and gram-negative species (24). Particularly in *B. subtilis*, it would appear that insertions in some hotspot chromosomal regions are much more frequent than in others (49). Even within these hotspot regions, however, insertions are distributed quite randomly, and insertions outside of hotspot regions are sufficiently abundant and random to permit very effective insertional mutagenesis (40, 49). Our results suggest that the same is true for *L. monocytogenes*. Although the distribution of insertional auxotrophic mutations was not completely random, many different kinds were recovered, even in this relatively limited study. Insertions within the *hlyA* gene are significantly more frequent than would be expected on a purely random basis, suggesting that this gene may be within...
one of the hotspot regions of the L. monocytogenes chromosome. In addition, as in B. subtilis and other bacteria (46), Tn917 appears to insert singly into the L. monocytogenes chromosome (data not shown).

Despite the large size of Tn917-LTV3 (15.5 kilobases), its ability to (i) insert randomly into the L. monocytogenes chromosome, (ii) form transcriptional fusions with lacZ, and (iii) allow the direct cloning of DNA adjacent to insertions should ensure its utility in future mutational analysis of L. monocytogenes. Similarly, Tn917-LTV1, which provides even greater flexibility in the choices of restriction enzymes for cloning adjacent DNA, should prove useful for transposon mutagenesis in a wide range of other gram-positive bacteria and particularly in species where this transposon derivative exhibits the high transposition frequency seen in B. subtilis.

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LITERATURE CITED

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