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Insertional Mutagenesis of *Listeria monocytogenes* with a Novel Tn*917* 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 Tn*917* were constructed, Tn*917*-LTV1 and Tn*917*-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 ColE1 replication functions, a cluster of polylinker cloning sites, and antibiotic resistance genes selectable in *E. coli* (*bla* in the case of Tn*917*-LTV1; *neo* and *ble* in the case of Tn*917*-LTV3). The enhanced transposition frequency of Tn*917*-LTV1 and Tn*917*-LTV3 (about 100-fold in *Bacillus subtilis*) is believed to be due to the fortuitous placement of vector-derived promoters upstream from the Tn*917* transposase gene. In *L. monocytogenes*, Tn*917*-LTV3 transposed at a frequency of 8×10^{-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 ColE1 *rep* functions and *neo* gene carried by Tn*917*-LTV3. Both insertions were shown to be within *hlyA*, the *L. monocytogenes* hemolysin structural gene. Although Tn*917*-LTV1 and Tn*917*-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, meningoencephalitis, septicimia, 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 Tn*1545* (11) and Tn*916* (15). Tn*1545* was delivered into *L. monocytogenes* at a frequency of approximately 10^{-8} through conjugation with strains of *L. monocytogenes* harboring this transposon (17), and Tn*916* was introduced through conjugation with Tn*916*-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 Tn*3*-like transposon Tn*917* 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 Tn*917*, 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 Tn*917* can function in *L. monocytogenes*, we have constructed two modified forms of the transposon, Tn*917*-LTV1 and Tn*917*-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 promoterless copy of the *Escherichia coli lacZ* gene orientated 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*, ColE1 replication functions, an M13 origin of replication, and a cluster of polylinker cloning sites. The polylinker 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 Tn*917* (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 Tn*917* insertions in *L. monocytogenes* and to test the utility of our modified derivatives of the transposon, several independent insertion libraries were obtained with Tn*917*-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 Tn*917* 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*::Tn*917*-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_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20*(Sm^r) *xyl-5 mtl-1 supE44* F^- λ^-] (5). The *E. coli* host for recovery of *L. monocytogenes* DNA sequences flanking transposon insertions was MC1061 [*hsdR mcrB araD139* Δ(*araABC-leu*)*7679* 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 pTV51 (50) by transforming *B. subtilis* PY339 containing pBD95Ts (46) to Tc^r with pTV51 DNA linearized by digestion with *Pst*I. 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 *Bam*HI-digested pTV51Ts and *Hin*dIII-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^r by using standard methods (34). To obtain pLTV3, the *bla* gene in pBG5 was replaced with the *neo* and *ble* genes from Tn*5* (26) prior to insertion into Tn*917-lac*. This was accomplished by insertion of the 1.9-kilobase *Hin*dIII-*Bam*HI fragment from Tn*5* containing its *neo* and *ble* genes into the 4.5-kilobase *Pvu*I-*Eco*RI 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^r transformants containing pBG5-*neo* were selected. Plasmid pBG5-*neo* was digested with *Hin*dIII, treated with Klenow fragment, and ligated in the presence of an equimolar amount of *Sma*I-digested pTV51Ts. To recover pLTV3, the ligation mixture was used to transform *E. coli* HB101, with selection for Tc^r 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 H_2O. 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 pancrease 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 $CaCl_2$ 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 $MgCl_2$ 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 $MgCl_2$ [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× SMM (SMM 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

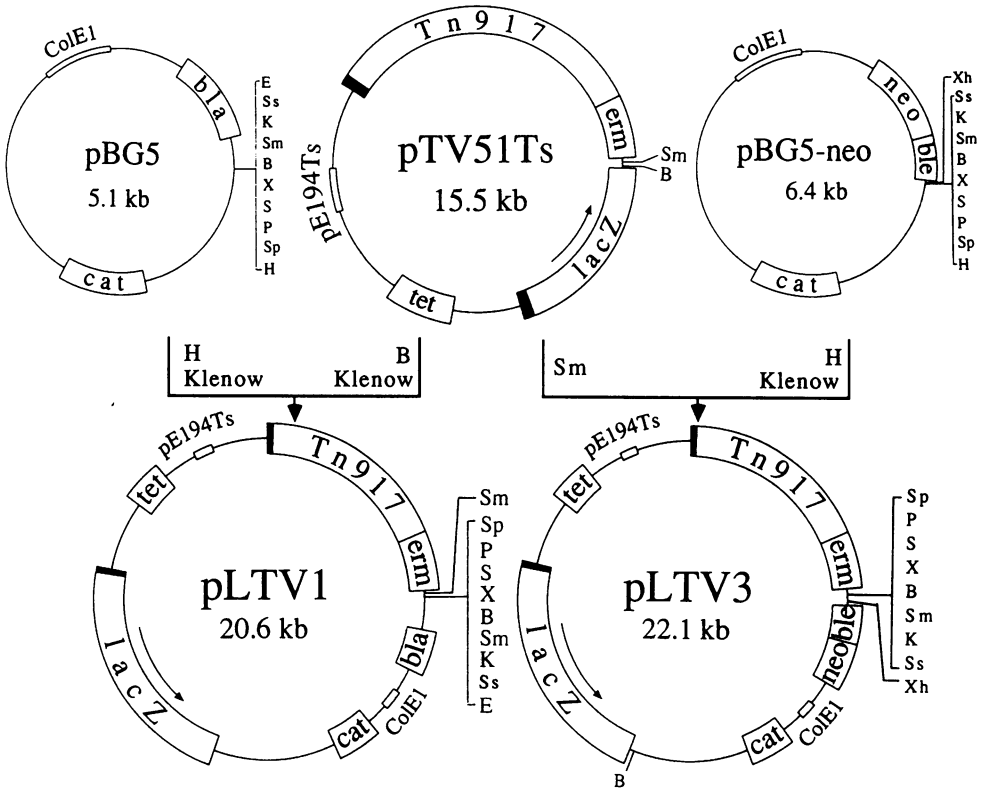

FIG. 1. The construction of pLTV1 and pLTV3. Plasmid pBG5 (50) is a ColE1-derived replicon which contains the pBR322 β-lactamase gene (*bla*) (4), M13mp19 polylinker (45), and *Staphylococcus aureus* pC194-derived chloramphenicol acetyltransferase gene (*cat*) (19). Plasmid pBG5-*neo* contains the neomycin phosphotransferase II (*neo*) and bleomycin (*ble*) determinants of Tn*5* (26) in place of the *bla* gene of pBG5. Plasmid pTV51Ts contains the pE194Ts temperature-sensitive replicon (41), the tetracycline resistance gene (*tet*) from pAMα1Δ1 (31), and Tn*917-lac* (32), which contains a promoterless *lacZ* gene from *E. coli* with translation initiation signals derived from *B. subtilis* gene *spoVG* (51) and Tn*917* ribosomal methytransferase gene (*erm*). Klenow refers to treatment of restriction fragments with the large subtilisn-generated fragment of *E. coli* DNA polymerase I (21) in the presence of deoxyribonucleoside triphosphates to produce blunt ends (43). Restriction endonuclease abbreviations used: *Eco*RI (E), *Sst*I (Ss), *Kpn*I (K), *Sma*I (Sm), *Bam*HI (B), *Xba*I (X), *Sal*I (S), *Pst*I (P), *Sph*I (Sp), *Hin*dIII (H), and *Xho*I (Xh).

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% K_2HPO_4, 0.15% KH_2PO_4, 0.5% glucose, 0.02 M $MgCl_2$, 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. An *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^r, Lm^r, and Tc^s, 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 *Xba*I, 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^r. Plasmid DNA was isolated from Km^r 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 Tn*917* 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 Tn*917*-derivatives in pLTV1 and pLTV3 were designated Tn*917*-LTV1 and Tn*917*-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 greater than 1,000-fold more β-galactosidase activity than the wild type.

To determine whether the insertion of pBG5 or pBG5-*neo* into Tn*917-lac* affected transposition, the transposition frequencies of Tn*917-LTV1* and Tn*917*-LTV3 in *B. subtilis* were determined and compared with an unaltered version of Tn*917* (Table 1). The transposition frequency of Tn*917* in *B. subtilis* was 5.8×10^{-5}, which was similar to that previously reported (47). Surprisingly, the transposition frequency of Tn*917*-LTV1 and Tn*917*-LTV3 in *B. subtilis* was approximately 100-fold greater. Transposition of Tn*917*-LTV3 in *L. monocytogenes* was approximately sevenfold less frequent than in *B. subtilis*, which was similar to that previously reported for Tn*917* (10).

Randomness of transposon insertion in *L. monocytogenes*. To evaluate the randomness of Tn*917* 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 Tn*917*-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.

TABLE 1. Transposition frequencies

Bacteria	Transposon (vector)[a]	Transposition frequency[b]
B. subtilis	Tn*917* (pTV1)	5.8×10^{-5} (± 5.0)
B. subtilis	Tn*917*-LTV1 (pLTV1)	5.5×10^{-3} (± 1.7)
B. subtilis	Tn*917*-LTV3 (pLTV3)	6.1×10^{-3} (± 4.6)
L. monocytogenes	Tn*917*-LTV3 (pLTV3)	8.2×10^{-4} (± 2.5)
B. subtilis	Tn*917-lac* (pTV51Ts)	2.0×10^{-4}[c]
B. subtilis	Tn*917-lac* (pTV32Ts)	8.0×10^{-5}[c]

[a] Vector refers to the plasmid on which each transposon was carried prior to transposition into the host chromosome.
[b] Mean and standard deviation of three separate determinations.
[c] One determination.

TABLE 2. Auxotrophic types

Transposon library	Auxotrophs isolated[a]
1	1 Ade, 1 Gly, 2 Pro, 1 Ura
2	3 Ade, 2 Ura
3	2 Ade, 1 Aro, 2 Gly, 1 Nic, 1 Phe, 1 Ura
4	9 Ade, 1 Gln, 1 Gly, 2 Pro, 1 Ura
5	6 Ade, 1 Ura
6	1 Ade, 1 Nic, 3 Ura
7	7 Ade, 2 Pro, 1 Ura
8	10 Ade, 1 Nic, 1 Phe, 1 Ura
9	5 Ade, 1 Pro, 1 Ura
10	4 Ade, 1 Gly, 1 Pro, 2 Ura

[a] Abbreviations: adenosine (Ade), aromatic early block (Aro), glutamine (Gln), glycine (Gly), nicotinic acid (Nic), phenylalanine (Phe), proline (Pro), and uracil (Ura).

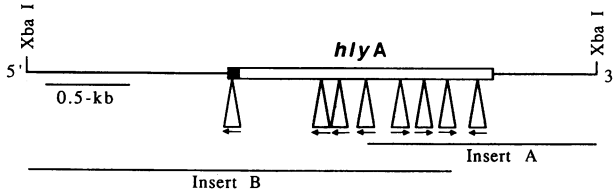

FIG. 2. Physical map of *L. monocytogenes hlyA* chromosomal region showing sites of Tn*917*-LTV3 insertion and cloned flanking DNA. Only sites for *Xba*I are shown. The solid portion of *hlyA* represents the predicted signal sequence (27). Locations of Tn*917*-LTV3 insertions are indicated below *hlyA* by open triangles. 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 *Xba*I, 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 ColE1 replicon within Tn*917*-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 *Xba*I. Because an *Xba*I site is present in the polylinker of Tn*917*-LTV3, sequences cloned by using *Xba*I 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 Tn*917-lac* containing ColE1 replication functions. By using one of these derivatives (Tn*917*-LTV3), we have shown that Tn*917* can insert into the *L. monocytogenes* chromosome with a relatively high degree of randomness, generating *lacZ* transcriptional fusions when insertions occur within genes in the appropriate orientation. The presence of ColE1 replication functions and polylinker cloning sites allowed the convenient and rapid cloning of flanking DNA. Unexpectedly, both derivatives, Tn*917*-LTV1 and Tn*917*-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 Tn*917* transposase is increased in these constructs due to transcriptional readthrough from within the inserted ColE1 replicons. In the case Tn*917*-LTV1, the *lac* promoter adjacent to the polylinker cluster in pBG5 (50) is positioned appropriately to direct transcription toward the Tn*917* transposase gene. This promoter is deleted in Tn*917*-LTV3 but is replaced by the promoter for the Tn*5* Sm^r 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 Tn*917*-LTV1 and Tn*917*-LTV3 differ only in the gram-negative antibiotic resistance marker contained within their ColE1-derived sequences. Although we would expect that the gram-negative *bla* gene present in Tn*917*-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 Tn*5* in the construction of Tn*917*-LTV3. Although Tn*917*-LTV3 might be the transposon of choice for other gram-positive pathogens as well (e.g., *Streptococcus* spp.), Tn*917*-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 Tn*917*-LTV1 and Tn*917*-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 Tn*917* 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

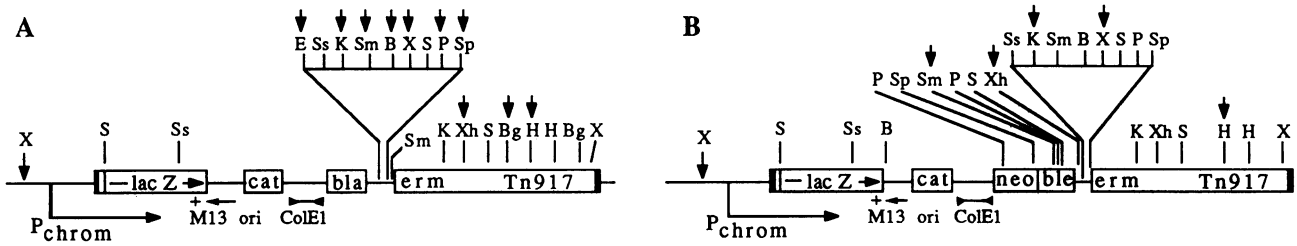

FIG. 3. Hypothetical chromosomal insertions of Tn*917*-LTV1 (A) and Tn*917*-LTV3 (B). In both cases, a transcriptional fusion between the transposon-containing *lacZ* gene and a chromosomal promoter is shown. Unique sites in or near the polylinkers, which can be used to clone adjacent sequence flanking the left end of the transposons, are indicated by vertical arrows. Restriction endonuclease abbreviations used: *Eco*RI (E), *Sst*I (Ss), *Kpn*I (K), *Sma*I (Sm), *Bam*HI (B), *Xba*I (X), *Sal*I (S), *Pst*I (P), *Sph*I (Sp), *Hin*dIII (H), *Xho*I (Xh), and *Bgl*II (Bg).

one of the hotspot regions of the *L. monocytogenes* chromosome. In addition, as in *B. subtilis* and other bacteria (46), Tn*917* appears to insert singly into the *L. monocytogenes* chromosome (data not shown).

Despite the large size of Tn*917*-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, Tn*917*-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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