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In Vivo Random Mutagenesis of *Bacillus subtilis* by Use of TnYLB-1, a *mariner*-Based Transposon

Yoann Le Breton,* Nrusingh Prasad Mohapatra,† and W. G. Haldenwang

Department of Microbiology and Immunology, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78229-3900

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This report describes the construction and characterization of a *mariner*-based transposon system designed to be used in *Bacillus subtilis*, but potentially applicable to other gram-positive bacteria. Two pUC19-derived plasmids were created that contain the *mariner-Himar1* transposase gene, modified for expression in *B. subtilis*, under the control of either σ^A- or σ^B-dependent promoters. Both plasmids also contain a transposable element (TnYLB-1) consisting of a Kan^r cassette bracketed by the *Himar1*-recognized inverse terminal repeats, as well as the temperature-sensitive replicon and Erm^r gene of pE194ts. TnYLB-1 transposes into the *B. subtilis* chromosome with high frequency (10^{-2}) from either plasmid. Southern hybridization analyses of 15 transposants and sequence analyses of the insertion sites of 10 of these are consistent with random transposition, requiring only a "TA" dinucleotide as the essential target in the recipient DNA. Two hundred transposants screened for sporulation proficiency and auxotrophy yielded five Spo$^-$ clones, three with insertions in known sporulation genes (*kinA*, *spoVT*, and *yqfD*) and two in genes (*ybaN* and *yubB*) with unknown functions. Two auxotrophic mutants were identified among the 200 transposants, one with an insertion in *lysA* and another in a gene (*yjzB*) whose function is unknown.

Bacillus subtilis has long been a subject organism for bacteriological research, serving as a model for analyses of the physiology of gram-positive bacteria and microbial differentiation (sporulation). Genetic analyses have been key to unraveling the biology of microorganisms, with transposon mutagenesis as a powerful tool in these analyses. Transposons can generate insertion mutations that are readily mapped and, depending on the particular transposon used, can also create reporter gene fusions at the sites of their insertion (3). The Tn*917* and Tn*10* transposons have been modified for use in *B. subtilis*. Each was successfully applied in a number of studies, but both have properties that limit their usefulness. Tn*917*, a streptococcal Tn*3*-like transposon, was the first transposon developed for use in *B. subtilis* (39). Although Tn*917* readily mobilizes into the *B. subtilis* chromosome, it does not insert randomly. Ninety-nine percent of all Tn*917* insertions occur at several "hot-spot" regions of the *B. subtilis* chromosome (40). As a result, large numbers of transposants need to be screened if a desired mutant is to be found among the 1% of insertions that occur randomly. The second *B. subtilis* transposon system was derived from the *Escherichia coli* transposon Tn*10* (22). Unlike Tn*917*, Tn*10* does not appear to have preferred insertion sites in the *B. subtilis* chromosome; however, it requires a particular 6-bp sequence as its target element (12). Unique 6-bp sequences occur randomly in DNA at approximately 4-kbp intervals (i.e., once in 4^6 bases). This reduces the number of potential Tn*10* insertion sites on the *B. subtilis* chromosome and, as a consequence, Tn*10*'s effectiveness as a tool for random mutagenesis.

The *mariner* transposable element *Himar1*, originally isolated from the horn fly *Haematobia irritans* (23), does not appear to have the limitations found in Tn*917* or Tn*10*. When coupled to appropriate expression elements, *mariner* transposons have been shown to insert randomly into the chromosomes of a number of bacterial species (4, 11, 17, 21, 24, 26, 34, 38). The *Himar1* transposase requires no obvious host factors to catalyze transposition, which occurs by a "cut-and-paste" reaction into the dinucleotide target "TA" (18). Given the positive attributes of the *mariner* system we undertook to adapt a *mariner*-based transposon for use in *B. subtilis*, using vectors and regulatory elements that might allow its eventual use in a number of gram-positive bacterial species.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. The *E. coli* strain DH5α and *B. subtilis* strain PY22 were cultured in Luria-Bertani (LB) medium (25) supplemented when necessary with the appropriate antibiotics (*E. coli*: ampicillin at 100 μg/ml, kanamycin [Kan] at 25 μg/ml, and erythromycin at 100 μg/ml; *B. subtilis*: kanamycin at 5 μg/ml and erythromycin at 1 μg/ml). Solid media contained 1.5% agar. Sporulation-deficient *B. subtilis* mutants were detected as colonies unable to produce brown pigment on Difco sporulation medium (DSM) (27) with kanamycin (5 μg/ml). Auxotrophic mutants were detected on minimal medium (29) supplemented with tryptophan (10 μg/ml) and kanamycin (5 μg/ml).

General molecular biology techniques. Plasmids were isolated using the Wizard *Plus* SV Minipreps kit (Promega, Madison, WI). Restriction enzymes (New England Biolabs, Beverly, MA), alkaline phosphatase (Roche, Indianapolis, IN), and T4 DNA ligase (New England Biolabs, Beverly, MA) were used according to the manufacturer's instructions. PCRs were performed using puRe Ready-To-Go PCR beads (Amersham Biosciences, Piscataway, NJ), 5 μg of DNA template, and 20 pmol of the appropriate primers (see Table 2). When necessary, PCR products were purified using the QIAquick PCR purification kit (QIAGEN,

* Corresponding author. Mailing address: Department of Microbiology and Immunology, MSC 7758, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900. Phone: (210) 567-3956. Fax: (210) 567-6612. E-mail: lebreton@uthscsa.edu.

† Present address: Department of Molecular Virology, Immunology and Medical Genetics, Department of Medicine, Division of Infectious Diseases, and The Centre for Microbial Interface Biology, Ohio State University, Columbus, OH 43210.

TABLE 1. Oligonucleotides used in this study

Purpose and name	Sequence[a] (5′–3′)	Use
Construction of TnYLB-1 delivery plasmids		
oTNP1	cgtcgac**taaggagg**tgagagcaATGGAAAAAAAGGAATTTCGTGTTTTGA-3′	*Himar1* allele cloning (SalI)
oTNP2	cgcgtcgacGCAGATTCCGGTCTAACAAAGAAAAAC	*Himar1* allele cloning (SalI)
oITR	ccccctgcagTAACAGGTTGGCTGATAAGTCCCCGGTCT	TnYLB-1 design (PstI)
oAtnpFwd	cccggtcaaTGGAGCAATTCGGACGATTGACAAGC	P_A-*Himar1* allele cloning (KpnI)
oAtnpRev	cccggtcaaGTCGACGCAGATTCCGGTCTAACAAAG	P_A-*Himar1* allele cloning (KpnI)
oOEFwd	cccgaattcGTCCAGAAGGTCGATAGAAAGCGT	pE194 OE cloning (EcoRI)
oOERev	cccgaattcCACTTGTAACCAGTCCGTCCACATGT	pE194 OE cloning (EcoRI)
IPCR experiments		
oIPCR1	GCTTGTAAATTCTATCATAATTG	IPCR amplification
oIPCR2	AGGGAATCATTTGAAGGTTGG	IPCR amplification
oIPCR3	GCATTTAATACTAGCGACGCC	IPCR DNA sequencing

[a] Nucleotides in lowercase are not complementary to the target sequence. Underlined nucleotides indicate sites inserted within primer sequences corresponding to the restriction enzymes reported in the right column. The newly created ribosome binding site sequence is in boldface.

Valencia, CA). *E. coli* strains were transformed by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories, Hercules, CA) as recommended by the manufacturer. Transformation of competent *B. subtilis* cells was carried out by the method of Yasbin et al. (36). Chromosomal DNA from *B. subtilis* strains was purified as previously described (13). Southern blot analyses were performed using a DIG High Prime DNA labeling and detection starter kit (Roche, Indianapolis, IN).

Detection of *mariner* transposition events. The *mariner* plasmids were transformed into *B. subtilis* strain PY22 selecting for Kan^r at 30°C. Transformant colonies were screened for plasmid-associated properties, i.e., Kan^r and Erm^r at the permissive temperature for plasmid replication (30°C) and Kan^r and Erm^s at the restrictive temperature (50°C). Plasmid DNA was then extracted (32) from the transformant clones with the appropriate phenotypes and subjected to restriction endonuclease analysis to verify that these clones contained the original intact plasmid. Representative plasmid-containing colonies were incubated overnight in liquid medium (LB) at 37°C. Samples were then plated on LB agar containing Kan and incubated at 50°C to select for transposants.

Mapping of transposon insertion sites. Five micrograms of transposant genomic DNA was digested with TaqI and then circularized in a ligation reaction using the "Rapid Ligation" kit (Roche, Indianapolis, IN) at a DNA concentration of 5 ng/μl. Ligation products were phenol extracted, ethanol precipitated, and resuspended in Tris-EDTA (TE) buffer at 10 ng/μl. Inverse-PCR (IPCR) was performed on 100 ng of ligated DNA with the puRe*Taq* Ready-To-Go PCR beads (Amersham Biosciences, Piscataway, NJ) using the primers oIPCR1 and oIPCR2, which face outward from the transposon sequence (Table 1). IPCR products were purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA) and sequenced using the primer oIPCR3 (Table 1).

DNA sequencing and sequence analysis. Plasmids or PCR fragments were sequenced by the Advanced Nucleic Acids Core Facility of the University of Texas Health Science Center at San Antonio. DNA sequence analyses were performed using the Mac Vector program (Kodak, Scientific Imaging Systems). The BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) and the SubtiList website (http://genolist.pasteur.fr/SubtiList) were used for database analysis (1, 20). The WebLogo program (http://www.bio.cam.ac.uk/cgi-bin/seqlogo/logo.cgi) was used to align the DNA sequence around the *mariner* insertion sites (28).

RESULTS AND DISCUSSION

Construction of transposon delivery vehicles. Transposition of *mariner* transposons occurs by a "cut-and-paste" mechanism, whose essential elements are the *Himar1* transposase, the inverse terminal repeat (ITR) sequence at which it excises the transposable element, and a "TA" dinucleotide insertion site in the target DNA (18). The transposase gene does not have to be within the ITR sequences to catalyze transposition of the DNA segment that lies between them. This allows construction of transposable elements in which the ITRs flank an antibiotic resistance cassette but not the transposase gene. Such assemblages transpose only the antibiotic resistance cassette which forms stable insertions when mobilized into target DNAs.

To construct a *mariner*-based transposon system for *B. subtilis*, the upstream and downstream ITRs of *Himar1-mariner* were amplified on a common DNA fragment from pMMOrf (19). A single oligonucleotide primer (oITR) (Table 1) that incorporated a PstI restriction endonuclease target sequence at its 5′ end was used. This generated unique PstI recognition sites at both ends of the amplified DNA. The amplified fragment was cloned into the PstI site of pUC19 (35), forming pITR. A Kan^r cassette, active in gram-positive bacteria, was cut from the streptococcal plasmid pUH1 (30) as a ClaI piece which, after being made "blunt" by Klenow DNA polymerase, was ligated into a SmaI site present between the two ITRs in pITR. The resulting plasmid (pTn1) carries TnYLB-1 (Kan^r cassette flanked by the *Himar1* recognition sequence). Next, a temperature-sensitive origin of replication and an erythromycin resistance cassette were amplified from the staphylococcal plasmid pE194ts (14) using oligonucleotides (oOEFwd and oOERev) (Table 1) that add EcoRI restriction sites to the fragment ends. This fragment was cloned into pTn1, forming pMarC (Fig. 1), a plasmid capable of replication in *B. subtilis* at 30°C but not 50°C and conferring both Erm^r and Kan^r on *B. subtilis* strains that carry it. To provide a source of *Himar1* transposase, a hyperactive allele of the *Himar1* gene (C9 mutant) was amplified from pBAD24 (19) using oligonucleotides oTNP1 and -2 (Table 1). The upstream oligonucleotide included a *B. subtilis* ribosomal binding sequence (31) placed 8 nucleotides upstream of the initiation codon of *Himar1*. Both oligonucleotides also included a SalI recognition site. The PCR product, cleaved with SalI, was cloned into the SalI site of pKA-16. pKA-16 is pUK19 (16) with a 370-bp EcoRI/BamHI PCR fragment encoding the P_A promoter of the *sigB* operon (33). After verifying that *Himar1* was in the correct orientation for expression from P_A, the P_A-*Himar1* portion of the plasmid was amplified using oligonucleotides oAtnpFwd and -Rev (Table 1) and cloned as a KpnI fragment into pMarC to form pMarA (Fig. 1).

To provide an alternative expression system for *Himar1*, the previously isolated hyperactive *Himar1* fragment was cloned

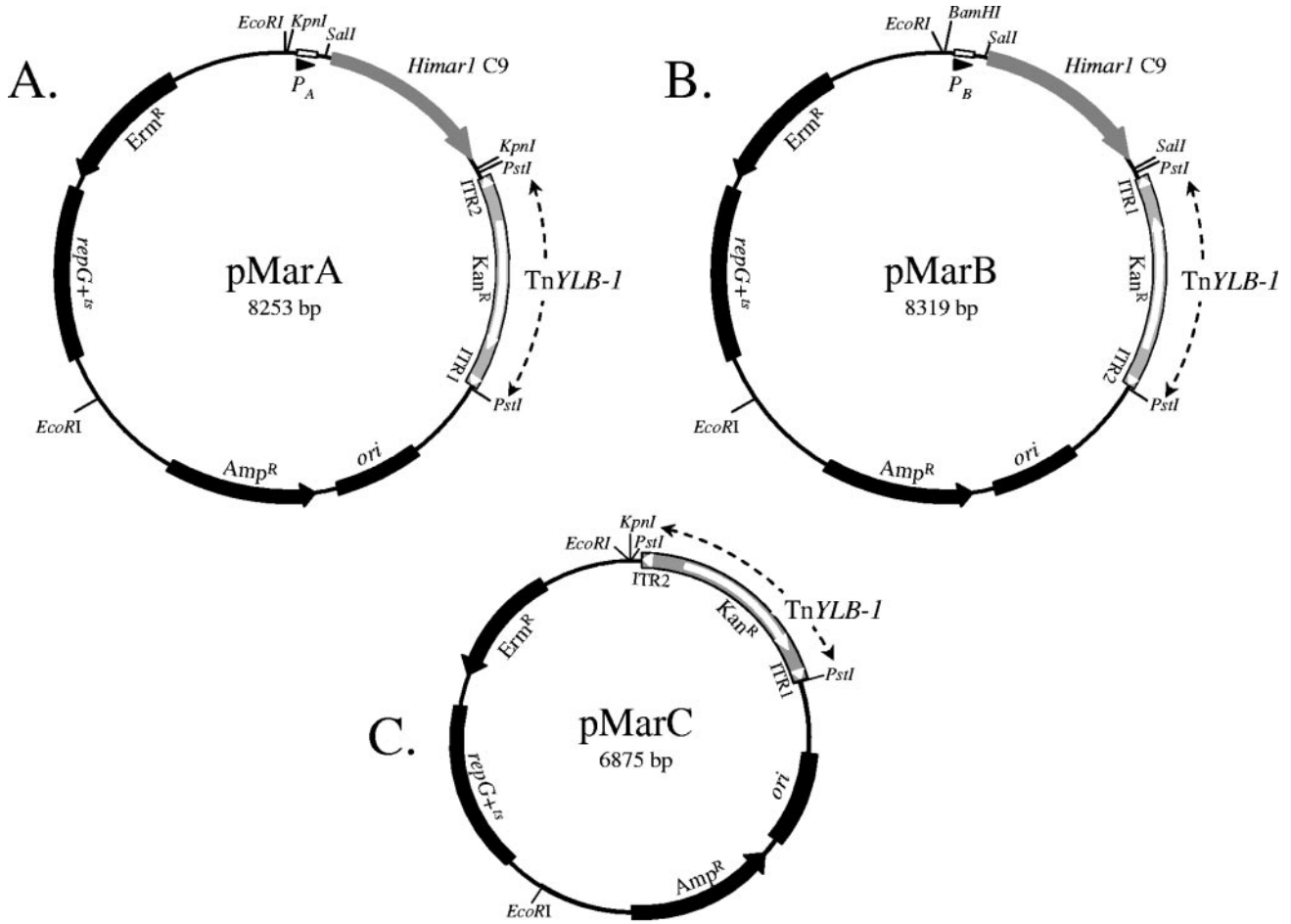

FIG. 1. Physical maps of the TnYLB-1 delivery plasmids. The TnYLB-1 transposon is contained in an *E. coli*/*B. subtilis* shuttle vector carrying a hyperactive allele of the *mariner-Himar1* transposase driven from a promoter recognized by σ^A (pMarA; A) or σ^B (pMarB; B) containing RNA polymerase. A control plasmid (pMarC; C) containing the TnYLB-1 transposon but lacking the transposase gene was also constructed. Kan^r, kanamycin resistance determinant; ori, pUC19 origin of replication; Amp^r, ampicillin resistance determinant; repG+^{ts}, pE194ts origin of replication; Erm^r, erythromycin resistance determinant from pE194ts; P_A and P_B, promoters recognized by σ^A-RNAP or σ^B-RNAP, respectively.

downstream of the stress-responsive σ^B promoter P_{ctc} (15), which had been previously cloned into pUC19 as a 339-bp BamHI/SalI fragment. The EcoRI fragment of pMarC containing the pE194ts replicon and the Erm^r cassette, as well as a PstI fragment encoding the Kan^r transposon, was then cloned into the P_{ctc}-*Himar1* plasmid to form pMarB (Fig. 1). As a result of these amplifications and clonings, two shuttle vectors were constructed that share the ability to replicate in *B. subtilis* at 30°C but not 50°C and encode Erm^r and Kan^r (TnYLB-1), which are selectable in *B. subtilis*. They differ in the promoters that drive the expression of the *Himar1* transposase gene. pMarA has *Himar1* under the transcriptional control of the *B. subtilis* housekeeping σ factor σ^A, while pMarB uses the general stress response σ factor σ^B for transposase expression.

***mariner* transposition in *B. subtilis*.** The *Himar1*-expressing plasmids pMarA and pMarB, as well as pMarC, a plasmid containing the Kan^r transposable element but not the transposase, were separately transformed into *B. subtilis* strain PY22 and plated on LB agar containing Kan (5 μg/ml) at 30°C. After 48 h individual colonies were tested as described in Materials and Methods for the presence of intact plasmids and the anticipated phenotypes.

Isolated clones were grown overnight in liquid LB medium at 37°C, and then portions of each culture were plated on either LB, LB plus 5 μg/ml Kan, or LB plus 10 μg/ml erythromycin (Erm) and incubated at the nonpermissive temperature for plasmid replication. Representative data that are the averages of two separate experiments are presented in Fig. 2A. Kan^r clones, representing likely transposition events, appeared with approximately equal frequencies ($\sim 10^{-2}$) regardless of whether the *Himar1* gene was expressed from the P_A or P_{ctc} promoter. No antibiotic-resistant clones were detected when *B. subtilis* carrying the plasmid (pMarC) lacking the transposase coding sequence was plated at 50°C. Approximately 90% of the thermoresistant Kan^r clones were sensitive to Erm, the antibiotic resistance encoded by the plasmid but not included within TnYLB-1. Erm^r clones could reflect either subpopulations of cells in which plasmid replication had become temperature resistant or transposition events from plasmid multimers in which plasmid sequences, including the Erm^r cassette and the

A.

Delivery plasmid used	Viable cell count (CFU/ml)			Transposition frequency	Erm^R/Kan^R
	LB 50°C	LB KanR 50°C	LB ErmR 50°C		
pMarA	$8(\pm 0.8) \times 10^8$	$6.2(\pm 1.3) \times 10^6$	$7.2(\pm 1.2) \times 10^5$	$8(\pm 0.52) \times 10^{-3}$	11(±1.1) %
pMarB	$6(\pm 0.5) \times 10^8$	$6(\pm 1.1) \times 10^6$	$2.5(\pm 0.6) \times 10^5$	$1(\pm 0.25) \times 10^{-2}$	4.1(±0.3) %
pMarC	$7(\pm 0.1) \times 10^8$	0	0	-	-

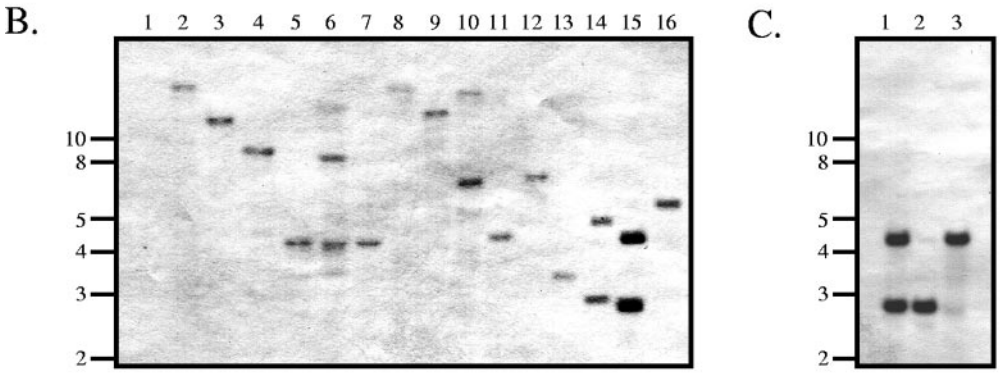

FIG. 2. TnYLB-1 transposition in *B. subtilis*. A. CFU resulting from plating of two separate overnight (O/N) cultures of *B. subtilis* strains carrying either pMarA, -B, or -C at 50°C on LB agar with or without the indicated antibiotics. Transposition frequency is calculated as Kan^r colonies/LB colonies. Erm^r/Kan^r represents the percentage of the O/N cultures that displayed the plasmid-encoded antibiotic resistance (Erm^r) versus the transposon-encoded resistance (Kan^r). B. Southern hybridization analysis of TnYLB-1 insertions in *B. subtilis*. Chromosomal DNA from wild-type *B. subtilis* PY22 (lane 1) or 15 Kan^r clones isolated at 50°C from PY22/pMarB (lanes 2 to 16) was digested with EcoRI and analyzed by Southern blotting as described in Materials and Methods, using a hybridization probe specific for TnYLB-1. DNA fragment sizes (kbp) are indicated to the left and are based on DNA markers included in the separating gel. C. Southern blot analysis of the separation of individual TnYLB-1 insertions from a multiple-insertion strain. Chromosomal DNA from a transposant harboring two transposon insertions (lane 1) was extracted and transformed into *B. subtilis* PY22 to yield transformants with single transposon insertions (lanes 2 and 3).

transposon, are inserted into the *B. subtilis* chromosome. In this circumstance, the *Himar1* transposase acts on ITR elements of the multimeric plasmids other than those that immediately bracket the Kan^r cassette. Transposition of larger plasmid sequences from multimeric plasmids following transpositions of both Tn*917* and Tn*10* in *B. subtilis* has been described (39, 22). No Kan^r or Erm^r clones were found when the *Bacillus* strain carrying a plasmid without the transposase gene (pMarC) was plated at 50°C (Fig. 2A). This argues that the Erm^r clones that we observe are likely a consequence of transposition and not a loss of plasmid temperature sensitivity.

Analysis of transposon insertions. To verify integration of TnYLB-1 into the *B. subtilis* chromosome and test whether the insertions are likely to be random, Southern blot analyses were performed on 15 Kan^r clones that had been isolated after plating *B. subtilis*/pMarB at 50°C. Chromosomal DNA was extracted from these clones and cut with EcoRI. EcoRI cuts pMarB twice, releasing a 4.5-kbp fragment containing the transposon but does not cut within the transposon itself. If the transposon had inserted randomly into the chromosomes in these 15 clones, a transposon-specific probe would be expected to hybridize to a single, uniquely sized DNA fragment, generated by the EcoRI cleavage of each clone's chromosomal DNA. Digoxigenin-labeled DNA specific for the transposon was created in a PCR amplification using the oligonucleotide primer that hybridizes to the transposon's ITR elements and pTn1 as the template. Hybridization of this probe to EcoRI-digested DNA from each of the 15 Kan^r clones yielded the patterns illustrated in Fig. 2B. Although most of the clones appeared to have single unique insertions, approximately one-third of the clones (Fig. 2B, lanes 6, 10, 14, and 15) displayed multiple bands. The presence of multiple bands suggests that some clones contain more than one transposon insertion. To determine whether the multiple TnYLB-1s are unlinked to each other and therefore likely to be the result of independent insertions at different sites on the chromosome, chromosomal DNA from one of the strains with multiple TnYLB-1 bands (Fig. 2B, lane 15) was extracted and transformed into wild-type *B. subtilis*. Each of the two putative transposon insertions present in the original strain was found to separate from the other in the Kan^r transformant population (Fig. 2C). Thus, multiple independent transposition events had likely occurred in some of the initial transposition isolates. Given the high frequency (10^{-2}) with which TnYLB-1 is mobilized to the *B. subtilis* chromosome, the presence of multiple insertions in the chromosome of a significant portion of the transposant population might be expected. Multiple transpositions in the plasmid-containing strain could complicate an initial mutant anal-

TABLE 2. DNA sequence analysis of *B. subtilis* insertional mutants obtained by TnYLB-1 transposition from pMarB

Mutants and clone	Sequence at TnYLB-1 insertion site[a]	Point of insertion[b]	Gene name[b]	Potential function, comments[d] (reference[s])
Randomly picked mutants				
M1	**TA**CATTCCCACCTTACTGAATT	290252	—[c]	Promoter region of the *lmrBA* operon (37)
M2	**TA**TGTAAGATAATCGTGTCATA	2995834	*ytqI*	Unknown function
M3	**TA**ACACAATAACAAGCCAGTTA	232223	*ybeC*	Similar to amino acid transporter
Sporulation-deficient mutants				
Spo1	**TA**CAATTATACAGTCGTTCATT	160032	*ybaN*	Similar to polysaccharide deacetylase
Spo2	**TA**GACGATCTTGCCTTTGACAC	1469826	*kinA*	KinA, histidine kinase involved in sporulation initiation (10)
Spo3	**TA**TAACTTGGTAGAGAATAATC	2614732	*yqfD*	Unknown function, *yqfCD* expression regulated by σ^E (7, 9)
Spo4	**TA**GCAGCTTCTTCTGACATCAG	3194357	*yubB*	Similar to bacitracin resistance protein
Spo5	**TA**TATATTGCCGTGTCCGGCAG	64338	*spoVT*	Sporulation AbrB-like transcriptional regulator (2, 6)
Auxotrophic mutants				
Aux1	**TA**TGTCCTTGTTTGCTGTGACA	1207388	*yjzB*	Unknown function
Aux2	**TA**TTTCTCCGCTAAATAGAGAG	2437373	*lysA*	Diaminopimelate decarboxylase (5)

[a] Boldface letters correspond to the TA dinucleotide target of the TnYLB-1 transposon, whereas the rest of the sequence corresponds to the region surrounding the ITR1 end of the TnYLB-1 transposon.
[b] The position of TnYLB-1 insertion and the name of the targeted gene is given in regard to the nomenclature provided on the SubtiList database (20).
[c] —, of TnYLB-1 insertion occurred outside any open reading frame.
[d] Potential function is given based on homology searches performed using the SubtiList (20) and the BLAST databases (1). If TnYLB-1 insertion occurred in previously described genes, relevant references are provided.

ysis; however, at least for transformable bacteria such as *B. subtilis*, the segregation of each insertion in subsequent rounds of transformation makes this drawback easy to circumvent. For example, the possibility of multiple transposon insertions occurring during a mutagenesis experiment could be reduced if the transposon mutagenesis did not involve mobilization of the transposon from a plasmid delivery vehicle, but rather transformation of the test bacterium with a chromosomal DNA library from a *Bacillus* strain in which TnYLB-1 had randomly inserted.

The high frequency of TnYLB-1 transposition and the apparent randomness of its insertion sites on the *B. subtilis* chromosome argue that it should be a relatively efficient tool with which to isolate insertion mutations. To test this notion, 200 temperature-resistant Kan^r clones were spotted onto a glucose minimum medium to screen for auxotrophic mutations and onto DSM to identify clones that had lost the ability to sporulate. Of the 200 clones spotted 2 (Aux1 and -2) failed to grow on the minimal media and 5 separate clones (Spo1 to -5) failed to form Spo^+ colonies on DSM. Subsequent transformations with DNA extracted from these clones verified that the phenotypes were the result of the transposon insertions. To identify the *B. subtilis* genes disrupted by the insertions, as well as to further characterize the insertion sites, chromosomal DNA was extracted from the seven clones with Spo^- or auxotrophic phenotypes, as well as three additional Kan^r clones (M1 to -3) chosen at random. These DNAs were used in an inverse PCR protocol that amplified the chromosomal DNA abutting the transposons' ITRs (Materials and Methods). The amplified DNAs were then sequenced and the sequences aligned with the annotated *B. subtilis* genome (www.genolist.pasteur.fr/SubtiList/) (Table 2). Each of the 10 transpositions that were examined resulted in an insertion at a unique location on the *B. subtilis* chromosome. Of the five Spo^- insertions, two lie in characterized sporulation genes (*kinA* and *spoVT*) (6, 10). A third insertion lies in a gene (*yqfD*) whose disruption blocks sporulation and which is part of an operon transcribed by the sporulation-specific sigma factor σ^E (7, 9). The remaining two Spo^- insertions are in open reading frames (*ybaN* and *yubB*) that have not been studied. The two insertions that led to auxotrophy were found to be in *lysA*, encoding diaminopimelate decarboxylase, an enzyme in the lysine biosynthetic pathway, and *yjzB*, a gene of unknown function. Of the three insertions that displayed neither Spo^- nor auxotrophic phenotypes, two were in open reading frames (*ytqI* and *ybeC*) with unknown functions and the third was in the promoter region of the lincomycin resistance operon (*lmrBA*) (38).

The sequence data also demonstrate that TnYLB-1 had inserted, as expected, at "TA" dinucleotides. To ask whether sequences other than the TA element itself contributed to the insertion at these 10 sites, the nucleotide sequences bordering the TA target were aligned (Fig. 3A) and analyzed for conserved nucleotides using the WebLogo program (www.bio.cam.ac.uk/cgi-bin/seqlogo/logo.cgi) (28). The graphic representation of the sequence conservations at the target sites (Fig. 3B) highlights the invariant TA target element but also suggests a preference for T or A nucleotides 5 bases to either side of the insertion site. Eight of the 10 insertions occurred at TA dinucleotides that had an A or T at both of these positions. All of the insertions had A or T at at least one of them. Aside for this possible A/T preference, there are no additional sequences conserved among the insertion sites.

The data argue that the *Himar1*-based transposon system described in this report is effective for transposon mutagenesis in *B. subtilis*. TnYLB-1 is mobilized into the *B. subtilis* chromosome at a frequency (10^{-2}) which is significantly higher than that reported for transposons Tn*917* and Tn*10* (10^{-6} and 10^{-4}, respectively), which are commonly used in *B. subtilis*. In

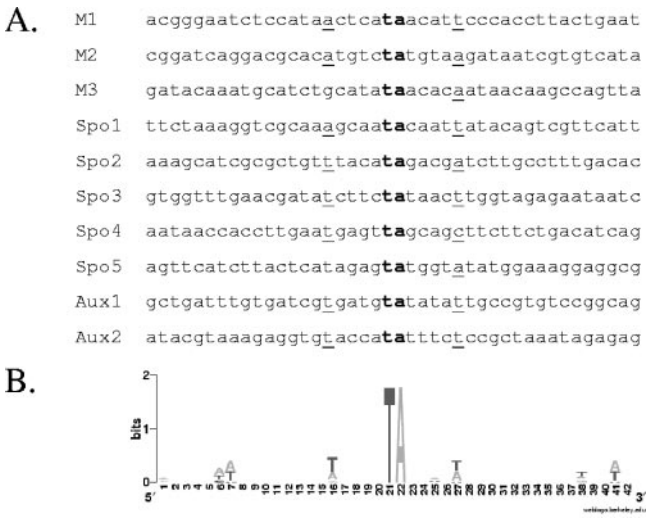

FIG. 3. Sequences surrounding the TnYLB-1 insertion sites. A. Sequences surrounding the TA insertion site (boldface) in three random Kan^r clones (M1 to M3), five Kan^r Spo$^-$ clones (Spo1 to -5), and two Kan^r auxotrophic clones (Aux1 and -2). The nucleotides at a position 5 bases to either side of the TA are underlined. B. "Web Logo" (http://www.bio.cam.ac.uk/cgi-bin/seqlogo/logo.cgi) consensus analysis of the transposon insertion site sequences (28). Nucleotide sequences at the site of *Himar1* insertion are displayed along the horizontal axis. Positions 21 and 22 correspond to the "TA" dinucleotide target. The height and order of each letter in the stack of letters at each position correspond to the relative frequency of the nucleotides at that position. Nucleotides used most frequently are on top. The total height of the stack is an indication of the conservation of a nucleotide at the particular site.

addition, its insertion into the *B. subtilis* chromosome appears to occur randomly, requiring a minimal consensus target sequence. This property should allow TnYLB-1 to be able to insert into any nonessential gene in *B. subtilis*. Aside from TnYLB-1's potential usefulness in *B. subtilis*, the transposon delivery systems described in this paper may also be useful for insertional mutagenesis in other gram-positive bacteria. The temperature-sensitive replicon and antibiotic resistances encoded on the plasmids are common to a number of gram-positive bacteria and, although the plasmid (pMarB) with a the σ^B-dependent promoter driving *Himar1* transcription would function only in bacterial species with a σ^B transcription factor (i.e., *Bacillus*, *Listeria*, and *Staphylococcus* spp.) (8), the plasmid (pMarA) with *Himar1* expression dependent on a sigma factor (σ^A) that is common to virtually all bacteria would be expected to have broader application.

pMarA, pMarB, and pMarC have been donated to the *Bacillus* Genetic Stock Center at the Department of Biochemistry, The Ohio State University, 484 West 12th Ave., Columbus, OH 43210 (www.bgsc.org).

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