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# Two novel transposon delivery vectors based on *mariner* transposon for random mutagenesis of *Bacillus thuringiensis*

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ABSTRACT

Two new *mariner* transposon delivery vector systems, pMarA333 and pMarB333, had been constructed to randomly mutagenize *Bacillus thuringiensis in vivo*. The results showed that both systems could randomly insert into the genome of *B. thuringiensis* YBT881 (CCAM 020673). These systems could have potential for further construction of mutant libraries of *B. thuringiensis* and other *Bacillus* strains.



*Bacillus thuringiensis* is a Gram-positive, endospore-forming bacterium that produces insecticidal crystal proteins which are the principal industrial biopesticide used in agriculture and insect vector control. Genetic analyses are the primary approaches to unraveling the biology of *B. thuringiensis*. With transposon mutagenesis as a powerful tool in these analyses many transposons, such as *Tn917*, *Tn916*, *Tn10* and *mariner*, are able to effectively insert into the genomes of certain bacteria and the insertion sites could be readily mapped. *Tn917* has been widely used in Gram-positive bacteria to construct insertion library (Bailey-Smith et al., 2005; Hoffmaster and Koehler, 1997). However, instead of randomly transposing into a genome, it has several insertion hot spots in chromosomes, especially in the replication terminus region. Besides, *Tn917* can also preferentially insert into plasmids and non-coding regions of a genome. *Tn916*, a conjugation transposon, has a stronger bias for non-coding regions than *Tn917* does (Garsin et al., 2004; Hoffmaster and Koehler, 1997). Therefore, a large number of transposants must be screened if a target mutant is to be found among insertion mutant library. *Tn10*, a tranposon from *E. coli*, has been widely applied in Gram-positive bacteria and some valuable genes had been found out by the use of *Tn10*-based transposons (Day et al., 2007; Espinasse et al., 2002; Fedhila et al., 2004; Gominet et al., 2001). However, it could not randomly insert into the chromosome, because it requires a symmetrical six-base-pair "GCTNAGC" as its target sequence (Pribil and Haniford, 2003). In addition, the homology between *Tn10* and its target, even distance from the site of the actual recombination event, could lead to the preference of *Tn10* (Monod et al., 1997). Furthermore, *Tn10*-encoded transposase contacts a large stretch of the target DNA (~24 bp) with a symmetrical structure, which also may contribute to the selection of targets (Pribil and Haniford, 2000). As a result, all these factors could reduce the effectiveness of selecting the target mutants and a large amount of mutants have to be screened in order to obtain the desired ones.

The *mariner* transposon, originally isolated from the horn fly *Haematobia irritans*, does not seem to have the limitations of *Tn917*, *Tn916* and *Tn10* (Le Breton et al., 2006). The *mariner* tranposon could insert into a target DNA by a "cut and paste" reaction, which is catalyzed by *mariner*-encoded transposase and requires no obvious specific host factors (Vos et al., 1996). It could randomly insert into target DNA with dinucleotide "TA" flanking it with $Mg^{2+}$ *in vitro* transposition, while in the presence of $Mn^{2+}$, there are no conserved flanking sequences (Lampe et al., 1996). Subsequently, it has been successfully applied in Gram-positive bacteria including *Bacillus* (Bae et al., 2004; Tam et al., 2006; Wilson et al., 2007). Given the positive attributes of the *mariner* system, we constructed two new transposon delivery vector systems based on *mariner* transposon, using vectors and regulatory elements that might allow their eventual use in *B. thuringiensis* along with other *Bacillus*.

For construction of pMarA333, the fragment containing pUC19-ori and spectinomycin-resistance cassette was amplified from pIC333 (Wilson et al., 2007) using primers oSO-F (atgcGACGTCTAA-CAGGTTGGCTGATAAGTCCCCGGTCTATTAATGAATCGGCCAACG) and oSO-R (cccAAGCTTTAACAGGTTGGCTGATAAGTCCCCGGTCTGGG-TAAACGCTGAATATCG). This fragment was nominated as ITRs_SO. The plasmid pMarA was digested with *Pst*I to remove the inverse terminal

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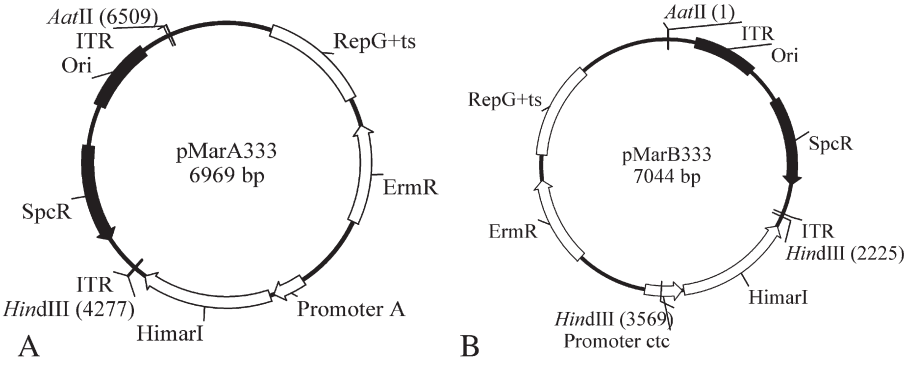


**Fig. 1.** Transposon delivery vectors. A. pMarA333, *HimarI* transposase was under control of $P_A$ promoter. SpcR, spectinomycin-resistance determinant; Ori, *E. coli* replication origion; RepG + ts, pE194ts origin of replication; ErmR, erythromycin-resistance determinant from pE194ts; HimarI, *HimarI* transposase; ITR, inverse terminal repeat recognized by HimarI transposase. B. pMarB333, *HimarI* transposase was under control of $P_{ctc}$ promoter.

repeats (ITRs) and kanamycin-resistance cassette, and self-ligated to construct pMarAs. pMarAs was then cut by *Aat*II and *Hin*dIII, and the fragment containing a hyperactive allele of the *Himar1* C9 mutant (Lampe et al., 1999), temperature-sensitive replicon (RepG + ts) and erythromycin-resistance cassette of pE194ts (Yan et al., 2008) was cloned into the ITRs_SO digested by *Aat*II and *Hin*dIII to construct pMarA333 (Fig. 1A).

Similarly, for construction of pMarB333, pMarB (Le Breton et al., 2006) was also cut by *Pst*I and self-ligated to construct pMarBs, which lost ITRs and the kanamycin-resistance cassette. pMarBs was digested by *Aat*II and *Hin*dIII, and the fragment containing temperature-sensitive replicon (RepG + ts) and Erm$^r$ cassette of pE194ts was cloned into the ITRs_SO digested by *Aat*II and *Hin*dIII to construct pMarC333. Then, the fragment containing the hyperactive allele of the *Himar1* gene (C9 mutant) was cut from pMarBs by *Hin*dIII and cloned into *Hin*dIII-digested pMarC333 to obtain pMarB333 (Fig. 1B) after verifying the fragment was ligated in the right direction. In pMarA333, the transposase was under the control of house-keeping promoter $P_A$, and in pMarB333, the transposase was on the downstream of the stress-responsive promoter $P_{ctc}$ (Le Breton et al., 2006). Comparing with pMarA and pMarB, the pUC19-ori was placed within the two ITRs, and made the circular fragments containing insertion sequence could replicate in *E. coli*, providing an easier method of detecting the transposon-flanking DNA sequence.

The *mariner*-based transposon system, pMarA333 and pMarB333, were separately transformed into *B. thuringiensis* YBT881 and the tramsformants were selected on Luria–Bertani (LB) broth agar containing Spc (300 μg/mL) and Erm (5 μg/mL) at 28 °C. After verifying the intact of the vectors, isolated clones containing pMarA333 or pMarB333 were cultured in LB for 6–8 h, and then portions of each culture were plated on LB/agar, LB/agar containing Spc (250 μg/mL) and LB/agar plus Erm (5 μg/mL) and then incubated for 24 h at 41 °C, a non-permissive temperature for the plasmids replication (Liang et al., 2007). In pMarA333-containing *B. thuringiensis*, transposition occurred in about 4% of viable cells as measured by spectinomycin resistance, while in pMarB333-containing *B. thuringiensis*, it occurred in a high frequency of approximate 15.7% (Table 1).

To test whether the insertions are likely to be random, 10 clones displaying Spc$^r$ and Erm-sensitive were randomly selected from pMarA333-inducing transposition collection and pMarB333-inducing collection, respectively. The total DNA was separately extracted from transposed mutant strains and digested by *Eco*RI, *Hin*dIII, *Sac*I, *Sal*I or *Nde*I, all of which couldn't cut within transposon sequence. The digested total DNA was purified by the use of a PCR Cleanup Kit (AXYGEN) and re-ligated to transform *E. coli* DH5α. Re-ligated DNA containing pUC19 replication origin and Spc$^r$ gene could allow the transformed clones to grow on LB/agar containing Spc (100 μg/mL). Transposon-flanking DNA was sequenced by the use of oMarSO (AAAGCGTCCTCTTGTGAAAT). DNA-sequencing revealed that all sequenced clones were the results of independent transposition events in either pMarA333-inducing or pMarB333-inducing transposition, suggesting *mariner* has no obvious bias in chromosome of *B. thuringiensis* YBT881 (Table 2). Besides, there is no same insertion site between pMarA333-indecued transposants and pMarB333-induced transposants, providing additional evidence that transposition occurs randomly. Therefore, both of the two transposon systems have been successfully applied in *B. thuringiensis* YBT881 and it could be an effective tool to construct insertion mutant library.

In this study, two *mariner*-based transposon were constructed to randomly mutagenize *B. thuringiensis* for the first time *in vivo*. The data argued that the *mariner*-based transposon delivery vectors described in this report were effective for transposon mutagenesis in *B. thuringiensis*. The plasmids pMarA and pMarB could not be applied in mutagenesis of *B. thuringiensis* YBT881, because *B. thuringiensis* YBT881 displayed kanamycin-resistance. In pMarA333 and pMarB333, kanamycin-resistance cassette was replaced with spectinomycin-resistance cassette, which could function in *B. thuringiensis* YBT881. Also, the pUC19-ori was placed within the two ITRs of the two transposon vectors, and allowed the circular fragments containing insertion sequence to replicate in *E. coli*, providing an easier and more accurate method of detecting the transposon-flanking DNA sequence. Compared with another *mariner*-based transposon vector pAW068 (accession no. EU146228), pMarA333 and pMarB333 have different temperature-sensitive replicon and promoters, which provided an alternative tool to construct an insertion

**Table 1**
Transposition activities of *mariner* transposon in *B. thuringiensis* YBT881.

| | Viable cell count(CFU/ml) | | | Transposition efficiency[a] | Plasmid clearance[b] |
|---|---|---|---|---|---|
| | LB | LB/Spc | LB/Erm | | |
| pMarA333 | $9.4(\pm 0.6)\times 10^6$ | $3.9(\pm 0.9)\times 10^5$ | $5.0(\pm 0.7)\times 10^4$ | 4.2(±0.9)% | 87.2(±1.8)% |
| pMarB333 | $2.8(\pm 0.4)\times 10^7$ | $4.4(\pm 0.1)\times 10^6$ | $9.0(\pm 2.0)\times 10^5$ | 15.7(±0.4)% | 79.5(±4.5)% |

Viable cell count data are the mean of 3 independent experiments and values in parentheses are standard deviation from the mean.
[a] Transposition efficiency is calculated as percentage of viable spectinomycin-resistant cells over total number of viable cells in absence of selection.
[b] Plasmid clearance is calculated as 100 minus percentage of viable erythromycin-resistant cells over viable spectinomycin-resistant cells.

**Table 2**
Analyses of insertion sites of randomly chosen *mariner* transposon mutants.

| Transposon number | Results of BLAST/GI: region, in or between ORF | Annotation | Identity (% base pairs) |
|---|---|---|---|
| *pMarA333-inducing transposants* | | | |
| BT881A003 | 218158707: 5,356,445 bp to 5,357,383 bp, in ORF | Bactoprenol glucosyl transferase | 98% in 170 bp |
| BT881A004 | 29899096: 4,690,732 bp to 4,691,877 bp, in ORF | Glycosyltransferase | 86% in 457 bp |
| BT881A007 | 118415003: 1,632,862 bp to 1,634,436 bp, in ORF | Flagellar M-ring protein | 92% in 632 bp |
| BT881A008 | 49328240: 3,847,973 bp to 3,848,815 bp, in ORF | Maltosaccharide ABC transporter, permease | 95% in 224 bp |
| BT881A009 | 218158707: 1,238,061 bp to 1,238,588 bp, in ORF | Conserved hypothetical protein | 96% in 507 bp |
| BT881A010 | 118415003: 1,230,497 bp to 1,231,033 bp, in ORF | Conserved hypothetical protein | 95% in 63 bp |
| BT881A012 | 118415003: 652,152 bp to 653,681 bp, in ORF | Cardiolipin synthetase 2 | 90% in 630 bp |
| BT881A019 | 118415003: 863,669 bp to 864,631 bp, in ORF | Permease, drug/metabolite transporter | 89% in 422 bp |
| BT881A021 | 49328240: 3,419,781 bp to 3,420,188 bp, between ORFs | probable transposase for IS150 | 87% in 861 bp |
| BT881A022 | 118415003: 4,782,887 bp to 4,783,780 bp, between ORFs | Conserved hypothetical protein | 100% in 203 bp |
| *pMarB333-inducing transposants* | | | |
| BT881B049 | 49328240: 5,164,970 bp to 5,166,271 bp, in ORF | Methyl-accepting chemotaxis protein | 89% in 933 bp |
| BT881B050 | 42740913: 3,821,589 bp to 3,822,971 bp, in ORF | Phage integrase family protein | 93% in 938 bp |
| BT881B051 | 49328240: 4,548,091 bp to 4,549,233 bp, in ORF | Glycosyl transferase, group 1 family | 95% in 597 bp |
| BT881B053 | 50346902, in ORF | IS231S transposase | 97% in 133 bp |
| BT881B054 | 29899096: 410,851 bp to 413,832 bp, in ORF | Peptide synthetase | 93% in 1076 bp |
| BT881B055 | 49328240: 942,875 bp to 944,464 bp, in ORF | Sensor histidine kinase | 89% in 655 bp |
| BT881B062 | 118415003: 1,477,289 bp to 1,478,041 bp, between ORFs | Sensor histidine kinase | 93% in 424 bp |
| BT881B063 | 49328240: 185,625 bp to 186,884 bp, in ORF | Drug resistance transporter Bcr/CflA subfamily | 90% in 1058 bp |
| BT881B064 | 218158707: 428,467 bp to 432,048 bp, in ORF | Linear Gramicidin synthetase subunit C | 99% in 370 bp |
| BT881B067 | 45685585, between ORFs | cry2Aa | 97% in 211 bp |

The plasmids pMarA333-inducing transposants are designated as BT881AXXX, and pMarB333-inducing transposants are designated as BT881BXXX. For insertions into the non-coding regions, the physically closest ORF is indicated. Insertions into ORFs, the ORFs are indicated. Because there is no information of the whole genome of YBT881, we could only infer the information of insertion sites by BLAST through the whole NCBI database.

mutant library and might drive more expression of *HimarI* transposase. Higher expression transposase can lead to higher transposition activity in some conditions (Lampe et al., 1998), and this might partly explain why both pMarA333 and pMarB333 showed higher transposition efficiencies than that of pAW068 in *B. anthracis*. Both of these transposon vectors have been used in our lab to find out a number of factors affecting expression of crystal proteins and virulence of *B. thuringiensis*. Up to now, these two transposon systems have been tested only in *B. thuringiensis* YBT881, and transposition efficiency may be different in other *B. thuringiensis* strains. Besides, the temperature-sensitive replicon and antibiotic resistances encoded on the plasmids are common to a number of Gram-positive bacteria. Therefore, either pMarA333 or pMarB333 could be expected to have broader application in Gram-positive bacterial strains.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mimet.2009.06.008.

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