Note

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

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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 transposon 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 transposon 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 Mg2+ in vitro transposition, while in the presence of Mn2+, 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-CAGGTTCGCTGATAAGTCCCAGGTTATTAATGAATCGGCCAACG) and oSO-R (cccAAGCTTTAACAGGTTGGCTGATAAGTCCCCGGTCTATTAATGAATCGGCCAACG) and oSO-R (cccAAGCTTTAACAGGTTGGCTGATAAGTCCCCGGTCTATTAATGAATCGGCCAACG). This fragment was digested as ITRs_SO. The plasmid pMarA was digested with PstI to remove the inverse terminal...
repeats (ITRs) and kanamycin-resistance cassette, and self-ligated to construct pMarAs. pMarAs was then cut by AatII and HindIII, 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 AatII and HindIII to construct pMarA333 (Fig. 1A).

Similarly, for construction of pMarB333, pMarB (Le Breton et al., 2006) was also cut by PstI and self-ligated to construct pMarB5, which lost ITRs and the kanamycin-resistance cassette. pMarB5 was digested by AatII and HindIII, and the fragment containing temperature-sensitive replicon (RepG+ts) and Erm' cassette of pE194ts was cloned into the ITRs SO digested by AatII and HindIII to construct pMarC333. Then, the fragment containing the hyperactive allele of the Himar1 gene (C9 mutant) was cut from pMarB5 by HindIII and cloned into HindIII-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 Pα, and in pMarB333, the transposase was on the downstream of the stress-responsive promoter PpEX (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 transformants 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% 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’ 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 EcoRI, HindIII, SacI, SstI or NdeI, 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’ 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 pMarSO (AAGAGCTGCTCTTGTGAAAT). DNA-sequencing revealed that all sequenced clones were the results of independent transposition events either in 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-induced 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

![Image](Image 133x608 to 471x741)

**Fig. 1.** Transposon delivery vectors. A. pMarA333, Himar1 transposase was under control of Pα promoter. SpeR, spectinomycin-resistance determinant; Ori, E. coli replication origion; RepG+ts origin of replication; ErmR, erythromycin-resistance determinant from pE194ts; Himar1, Himar1 transposase; ITR, inverse terminal repeat recognized by Himar1 transposase. B. pMarB333, Himar1 transposase was under control of PpEX promoter.

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

<table>
<thead>
<tr>
<th>Transposition efficiencya</th>
<th>Plasmid clearanceb</th>
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<td>pMarA333: 4.2(±0.9)%</td>
<td>87.2(±1.8)%</td>
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<tr>
<td>pMarB333: 15.7(±0.4)%</td>
<td>79.5(±4.5)%</td>
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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.
mutant library and might drive more expression of Himar1 transposase. Higher expression transposase can lead to higher transposition activity in some conditions (Lampe et al., 1998), and this might partly explain why both pMAR333 and pMAR333 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 replication and antibiotic resistances encoded on the plasmids are common to a number of Gram-positive bacteria. Therefore, either pMAR333 or pMAR333 could be expected to have broader application in Gram-positive bacterial strains.

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