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New transposon delivery plasmids for insertional mutagenesis in *Bacillus anthracis*

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Abstract

Two new transposon delivery vector systems utilizing Mariner and mini-Tn10 transposons have been developed for *in vivo* insertional mutagenesis in *Bacillus anthracis* and other compatible Gram-positive species. The utility of both systems was directly demonstrated through the mutagenesis of a widely used *B. anthracis* strain.

Bacillus anthracis is a Gram-positive, endospore-forming bacterium that is the etiological agent of anthrax. Full virulence in mammalian hosts requires the presence of both the anthrax toxin and capsule, each encoded by genes carried by separate virulence plasmids. Expression of toxin and capsule genes occurs through a complex, multi-factorial regulatory cascade (recently reviewed in (Fouet and Mock, 2006)). The complete regulatory circuit required for virulence control in *B. anthracis* is incompletely understood as several regulatory inputs known to control toxin and capsule gene expression currently have no mechanistic basis. Elucidation of virulence factor regulatory mechanisms has been hampered by the paucity of genetic tools available for use in *B. anthracis*. A robust system for random mutagenesis in *B. anthracis* would be of great value in identifying previously unappreciated regulatory elements that control both virulence and metabolic pathways.

Transposable genetic elements have been widely used as genetic tools across a broad range of microbial systems (Hayes, 2003). Transposons are particularly useful for random insertional mutagenesis to identify previously unknown regulatory and metabolic pathways. In fact, transposon mutagenesis was used in some early studies identifying virulence regulators of *B. anthracis* (Hoffmaster and Koehler, 1997, Koehler et al., 1994, Welkos, 1991). However, the Tn-917 transposon used in these studies preferentially inserted into virulence plasmid sequences over genomic sequences (Hoffmaster and Koehler, 1997), making Tn-917-based transposon mutagenesis inefficient in targeting non-plasmid DNA sequences.

We have developed two new transposon delivery vector systems that overcome the limitations of existing in vivo mutagenesis tools: the first based on the prokaryotic mini-Tn10 transposon and the second based on the eukaryotic Mariner transposon. The mini-Tn10 system has been used widely for random insertional mutagenesis in Gram-positive bacteria, including in *B. subtilis* (Dartois et al., 1996, Steinmetz and Richter, 1994). An improved and stabilized mini-Tn10 system has been constructed specifically for *B. anthracis*. Despite the advantage of long

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experience using Tn10 transposons in Gram-positive bacteria, mini-Tn10 transposons can insert non-randomly due to interference with endogenous Tn-type transposons (Craig, 1997, Van der Auwera and Mahillon, 2005). In contrast, a Mariner-based transposon system would be more likely to insert randomly due to lower site specificity and would face a lesser chance of endogenous transposon interference (Rubin et al., 1999). In order to compare the advantages of each system, both were built and compared empirically.

The components necessary for Tn10 transposition are encoded on a single plasmid, pAW016 (Figure 1A), which is a derivative of the widely used pIC333 Gram-positive transposon vector system (Steinmetz and Richter, 1994). pAW016 was created by amplifying the chloramphenicol cassette of plasmid pJM105A (Perego, 1993) using oligonucleotides oCAT5 (5′-AGGGAGCTCCGGCAATAGTTACCCTTATTATCA) and oCAT6 (5′-ACAATGCATTCGATGATCTGGAGCTGTAATATA) and cloning this amplified fragment into NsiI and SacI digested pIC333 (Steinmetz and Richter, 1994), thereby substituting the chloramphenicol-resistance cassette for the erythromycin-resistance cassette in pIC333. The transposon contains a spectinomycin resistance cassette for positively selecting transposed bacteria and a pUC origin of replication for rescue and sequencing of transposed sequences in *E. coli*. The transposase enzyme has been physically separated from the transposed sequence, resulting in stable transposon integration following removal of the plasmid backbone. The inducible P*tac* promoter directs transcription of the transposase gene (Bender and Kleckner, 1992), and, therefore, we have found that pAW016 stability in *E. coli* is substantially increased by propagating the plasmid in a *lacI*^{q} strain, such as SCS110, which represses transposase expression. To facilitate plasmid removal following transposition, the plasmid backbone contains a temperature sensitive origin of replication, functional in both Gram positive and negative bacteria. This origin is permissive for plasmid replication at 28°C and non-permissive above 35°C, facilitating removal of plasmid following transposition simply by incubating transposed cultures at 37°C. This offers a significant advantage over other temperature sensitive vectors that require temperatures in excess of 42°C, temperatures at which virulence plasmids pXO1 and pXO2 can be spontaneously lost (Ireland and Hanna, 2002, Marston et al., 2005). The presence on the plasmid backbone of the chloramphenicol selectable marker allows screening for loss of plasmid. The sequence of pAW016 has been deposited in GenBank (accession no. EU146227).

Similarly, all components necessary for Mariner transposition are carried on a single plasmid, pAW068 (Figure 1B). pAW068 was created by the addition of a blunt-ended BamHI fragment of pAW016 to MscI and SnaBI digested pSC189 (Chiang and Rubin, 2002) to create the pAW067 cloning intermediate. pORI-Cm (Brunsing et al., 2005) was digested with SmaI and cloned into PvuI-digested pAW067 that had been blunt-ended to generate pAW068. Like pAW016, the plasmid backbone of pAW068 carries the temperature sensitive pWVO1 origin of replication and a chloramphenicol selectable marker. Transposition is catalyzed by the hyperactive C9 variant of *Himar1* transposase (Lampe et al., 1999). The transposon sequence is derived from the *E. coli* transposon vector pSC189 (Chiang and Rubin, 2002), modified to contain a spectinomycin cassette active in a wide range of Gram-positive bacteria and a pUC Gram-negative origin of replication. The transposon also carries flanking FLP-recombinase target sites and FLAG tag allowing pAW068 to be used to globally epitope tag *B. anthracis* genes (Figure 1C). The sequence of pAW068 has been deposited in GenBank (accession no. EU146228).

The Tn10- and Mariner-based transposon systems were tested for transposition activity in *B. anthracis*. pAW016 and pAW068 were electroporated into *B. anthracis* strain 34F2 (pXO1^{+}, pXO2^{-}), and the plasmid-containing strains were screened for plasmid loss at non-permissive temperature. After testing of multiple transposition protocols, an optimized scheme for transposition was developed for each system. For the experiments presented here, 34F2

carrying either pAW016 or pAW068 was grown in Luria-Bertani broth (LB) at the permissive temperature of 28°C overnight. Cultures were then serially diluted, plated on LB/agar, LB/agar plus 100 μg/ml spectinomycin, and LB/agar plus 7.5 μg/ml chloramphenicol, and incubated at the non-permissive temperature of 37°C overnight. Results of test transpositions are presented in Table 1. In pAW016-containing *B. anthracis*, transposition occurred in just over 1% of viable cells as measured by spectinomycin-resistance, and, of these spectinomycin-resistant cells, the vast majority also lost non-transposon plasmid backbone sequence as measured by loss of chloramphenicol-resistance. In pAW068-containing *B. anthracis*, transposition occurred at a lower frequency than with the Tn10 system, but plasmid loss was nearly complete. These data suggest that both the Tn10 and Mariner transposon delivery systems function with high efficiency in *B. anthracis*.

To confirm the transposition activity of the Tn10 and Mariner systems, eight spectinomycin-resistant, chloramphenicol-sensitive colonies were selected from a single transposition collection. Genomic DNA was extracted from transposed mutant strains using the UltraClean Microbial DNA Isolation Kit (MoBio, Carlsbad, CA). The site of insertion was identified by restriction digestion of genomic DNA using a panel of restriction enzymes, including SalI, NsiI, EcoRI, and SacI. The enzymes BamHI, ClaI, NdeI, PstI, and XbaI should not be used as these common enzymes cut within the transposon sequence. Digested genomic DNA was then re-ligated and used to transform *E. coli*. The presence of the pUC origin of replication allows re-ligated DNA containing the transposed sequence to replicate in *E. coli* as a spectinomycin-resistant plasmid. Sequencing of transposon-flanking DNA was performed using transposon-specific primers oMarSeq1 for Mariner mutants (5′- GCTTGTCATCGTCATCCTTGT) and TSP3E for mini-Tn10 mutants (5′- ATATTCACGGTTTACCAC). DNA sequencing revealed that all 16 sequenced clones (8 each for Tn10 and Mariner systems) are the result of independent insertion events (Table 2). 15 of 16 insertion events occurred in the main chromosome while one insertion mapped to virulence plasmid pXO1, suggesting neither the Mariner nor Tn10 systems preferentially inserts into plasmid sequences as do Tn917-based transposon systems (Hoffmaster and Koehler, 1997). Further, as part of an on-going insertional mutagenesis screen, an additional 23 transposon mutants were analyzed by Southern blotting of restriction-digested genomic DNA. Each of the 23 mutants contains a uniquely-sized insertion, providing additional evidence that transposition is occurring randomly. To date, both transposon delivery vectors have been tested only in derivatives of the *B. anthracis* 34F2 strain, therefore, it is possible that transposition efficiency may vary in other *B. anthracis* strains.

The complementary nature of these two robust transposon systems has already contributed significantly to ongoing research into *B. anthracis* pathogenesis. Both of these transposon systems have been put to use in our lab to identify a number of previously unknown regulatory pathways affecting expression of *B. anthracis* virulence factors. Results of such studies are currently under investigation and should be published shortly. Further, both the Tn10 and Mariner constructs are fully functional in *B. subtilis* with transposition efficiencies similar to those obtained in *B. anthracis* (approximately 1% following a single overnight incubation), suggesting that both constructs could be used to perform in vivo mutagenesis of a wide range of Gram-positive bacteria.

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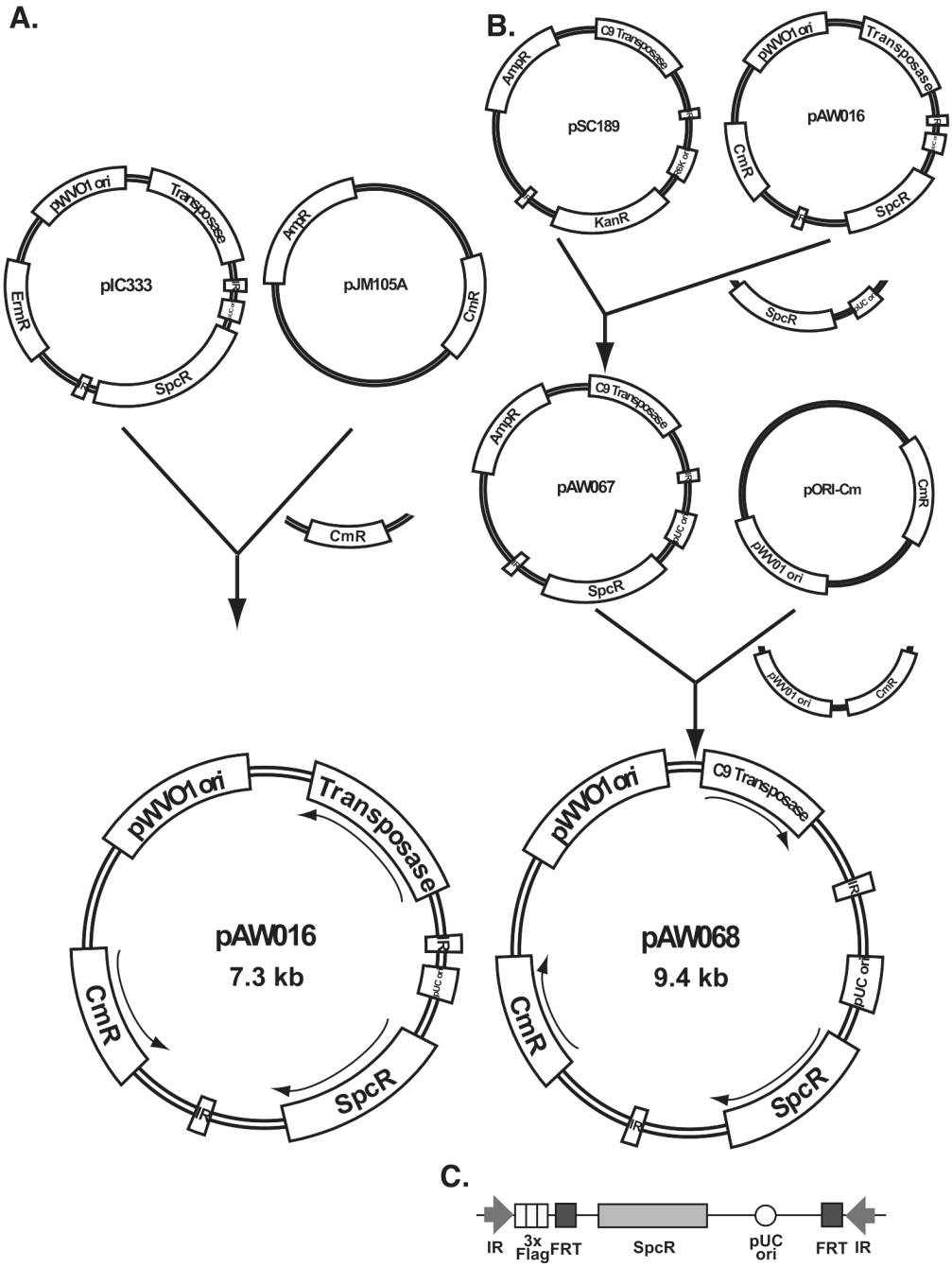

Figure 1.

Transposon delivery plasmids. **A**. pAW016, the mini-Tn10 delivery plasmid. CmR, chloramphenicol-resistance cassette; SpcR, spectinomycin-resistance cassette; IR, invert repeat transposase recognition sites. Vector constructed from source plasmids as indicated in text. **B**. pAW068, the Mariner transposon delivery plasmid. **C**. Features of the Mariner transposon sequence in greater detail showing the relative location of the pUC origin of replication, spectinomycin resistance cassette, flanking FLP recombinase target (FRT) sites, and the 3xFLAG epitope tag.

Table 1

Transposition activity of Mariner and mini-Tn10 transposon delivery vectors. Viable cell count data are the mean of 4 independent experiments and values in parentheses are standard deviation from the mean.

	Viable Cell Count (CFU/ml)			Transposition efficiency[a]	Plasmid clearance[b]
	LB	LB/Spc	LB/Cm		
pAW016 (mini-Tn10)	$1.8(\pm0.3) \times 10^8$	$2.4(\pm0.9) \times 10^6$	$2.3(\pm0.8) \times 10^3$	1.33(±0.35)%	98.9(±0.7)%
pAW068 (Mariner)	$2.4(\pm0.5) \times 10^8$	$7.6(\pm0.6) \times 10^5$	$2.2(\pm0.9) \times 10^2$	0.32(±0.09)%	99.9(±1.1)%

[a] Transposition frequency 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 chloramphenicol-resistant cells over viable Specinomycin-resistent cells.

Table 2

Insertion sites of randomly chosen mini-Tn10 and Mariner transposon mutants. mini-Tn10 insertions are designated Tn10, and Mariner insertions are designated Mar. For insertions into non-coding regions, the physically closest ORF is indicated. Insertion into ORF or insertion distance and direction from ORF as indicated in location column.

Transposon Number	Locus Number	Annotation	Location
Tn10 1	BAS3058	spermine/spermidine acetyltransferase, putative	ORF
Tn10 2	BAS2628	hypothetical protein	5′ 386 nt
Tn10 3	BAS2946	conserved hypothetical protein	3′ 59 nt
Tn10 4	BAS3113	conserved hypothetical protein	ORF
Tn10 5	BAS3656	zinc protease, insulinase family	ORF
Tn10 6	BAS4015	conserved hypothetical protein	ORF
Tn10 7	BAS1277	hypothetical protein	ORF
Tn10 8	BAS3430	hypothetical membrane protein	ORF
Mar1	BAS4928	hypothetical protein	ORF
Mar 2	BAS1610	ABC transporter, ATP-binding protein	ORF
Mar 3	BAS3609	conserved domain protein	ORF
Mar 4	BAS0657	conserved domain protein	ORF
Mar 5	BAS0792	sugE protein, putative	3′ 23 nt
Mar 6	BAS2474	conserved hypothetical protein	5′ 274 nt
Mar 7	BAS2922	conserved hypothetical protein	3′ 92 nt
Mar 8	pXO1-78	conserved hypothetical protein	ORF