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NOTE

Improved Antibiotic Resistance Gene Cassette for Marker Exchange Mutagenesis in *Ralstonia solanacearum* and *Burkholderia* Species

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Marker exchange mutagenesis is a fundamental approach to understanding gene function at a molecular level in bacteria. New plasmids carrying a kanamycin resistance gene or a trimethoprim resistance gene were constructed to provide antibiotic resistance cassettes for marker exchange mutagenesis in *Ralstonia solanacearum* and many antibiotic-resistant *Burkholderia* spp. Insertion sequences present in the flanking sequences of the antibiotic resistance cassette were removed to prevent aberrant gene replacement and polar mutation during mutagenesis in wild-type bacteria. Plasmids provided in this study would be convenient for use in gene cassettes for gene replacement in other Gram-negative bacteria.

***Keywords*:** antibiotic resistance, kanamycin, marker exchange, *Ralstonia solanacearum*, trimethoprim

Antibiotic resistance genes flanked at both ends by several identical restriction enzyme sites are useful for construction of insertionally mutated genes that can be used for marker exchange mutagenesis in bacteria of interest. Several plasmids carrying antibiotic resistance gene cassettes have been described (Vieira and Messing, 1982; Musso and Hodam, 1989; Murillo *et al*., 1994; Reece and Philips, 1995). An antibiotic resistance gene cassette flanked by symmetrical restriction sites in a high-copy number plasmid is convenient for use as a gene cassette with a single restriction enzyme.

Many antibiotics are not effective against some bacteria such as *Burkholderia cepacia* complex and *Burkholderia* spp. (Beckman and Lessie, 1979; Lewin *et al*., 1993). This is a limitation for studying bacterial gene function using gene-specific mutation in many environmental strains and in clinical strains of *B. cepacia* complex. However, trimethoprim is an effective antibiotic against many strains of *Burkholderia* spp. and is frequently used with sulfamethoxazole to suppress antibiotic-resistant bacteria (Peeters *et al*., 2009). The gene for trimethoprim resistance (Tp^R) from R-factor R388 encodes dihydrofolate reductase and is available as a selection marker (Amyes and Smith, 1976). In this study, we provide a Tp^R gene cassette that can be used to create a nonpolar mutation by marker exchange mutagenesis and can also be used in trimethoprim-sensitive *Burkholderia* spp.

A number of antibiotic resistance cassettes have been used for marker exchange mutagenesis. Many of them were derived from transposons (Oka *et al*., 1981; Musso and Hodam, 1989; Murillo *et al*., 1994), which contain the flanking sequences of the insertion sequence (IS) elements. These IS elements flanking the antibiotic resistance cassettes can result in a polar mutation during marker exchange mutagenesis (Musso and Hodam, 1989) owing to the presence of a transcriptional termination signal. In many cases, a nonpolar mutation in a single open reading frame is essential to analyze the function of the gene in bacteria.

The flanking IS elements can cause not only a polar mutation but also aberrant recombination in some wild-type bacteria. Our efforts to mutate some genes in a plant pathogenic bacterium, *Ralstonia solanacerum*, produced an aberrant recombination (Fig. 1), probably due to the presence of the flanking sequences of the kanamycin resistance (Km^R) gene (Fig. 2). To understand the role of extracellular polysaccharide (EPS) production in *R. solanacearum* during bacterial long-term survival, we attempted to generate a mutant defective in bacterial EPS production. EPS is a well-known bacterial virulence factor of *R. solanacearum* (Kao *et al*., 1992), but its function in relation to bacterial long-term survival in the soil has not yet been evaluated. Therefore, the gene *epsD* encoding NDP-N-acetyl-D-galactosaminuronic acid dehydrogenase, was amplified from *R. solanacearum* SL341 (Jeong *et al*., 2007) using two primers, ED-1 (5′-TTCGGGATCCTCGGTCAGCGGAAGCAC-3′) and ED-2 (5′-ACCAGGAATTCCCTGACGGGCGTGAAG-3′). The *epsD* gene was subsequently mutated by inserting the Km^R cassette (digested with *Eco*RI and Klenow-filled) from pMKm (Murillo *et al*., 1994) at the internal *Eco*RV site of *epsD*. Conventional marker exchange mutagenesis (Yang *et al*., 1996) was performed in the highly virulent wild-type *R. solanacearum* strain SL341, and several non-mucoid mutants were obtained. Southern blot analysis of digested genomic DNA from *R. solanacearum* strains was performed. A 1.0 kb segment of the *Pst*I/*Hin*dIII fragment of pMKm (Murillo *et al*., 1994) carrying a kanamycin resistance cassette and 977-bp segment of the *Sal*I fragment of *epsD* were used as probes to verify the correct gene replacement

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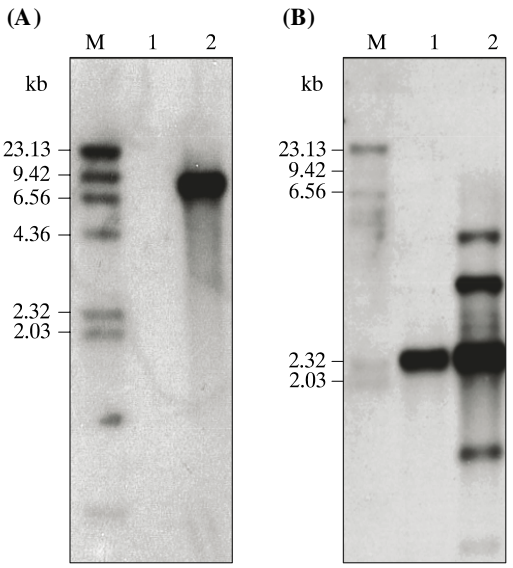


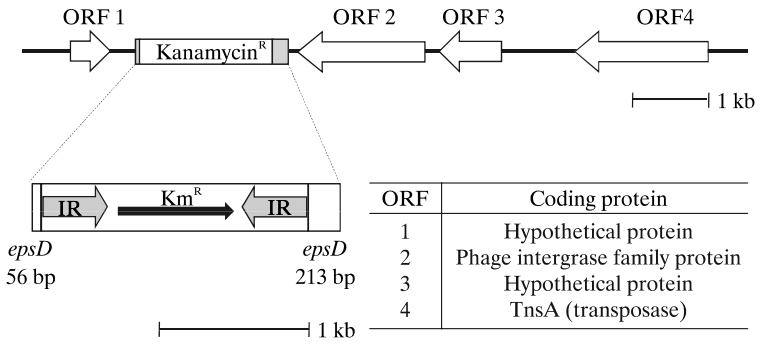
Fig. 1. Southern hybridization with genomic DNA of *R. solanacearum* SL341 and SL341E. Bacterial genomic DNA was digested with *Eco*RI for detection of the kanamycin resistance gene and with *Pst*I for detection of the *epsD* gene. (A) Hybridization with the kanamycin resistance gene probe. (B) Hybridization with the *epsD* gene probe. Lanes: M, DIG-labeled λ-DNA digested with *Hind*III; 1, genomic DNA of *R. solanacearum* SL341; 2, genomic DNA of SL341E (non-mucoid mutant).

of the *epsD* locus in *R. solanacearum* SL341 with *epsD::km*. The probes were labeled with a digoxigenin-dUTP DNA Labeling kit (Roche, Germany) and detected with a chemiluminescent substrate, CSPD, as described in the manufacturer's instructions. A single copy of the Km^R gene had inserted into the genome of SL341 strain, as verified by Southern hybridization (Fig. 1A). However, the gene replacement was found to have occurred at a different location in the mutant SL341E rather than in the *epsD* gene. Moreover, additional hybridization signals were detected in the SL341E genomic DNA (Fig. 1B). Other mutants that were obtained independently also showed the same pattern of gene replacement (data not shown), which suggests that an unexpected aberrant recombination occurred in the SL341 strain during marker exchange mutagenesis.

To analyze the aberrant recombination, the chromosomal locus of SL341E containing the mutational construct was cloned in pUC119 by shotgun library construction, clones were selected on kanamycin-containing medium, and the cloned DNA fragments were completely sequenced. DNA sequence analysis of the recombined chromosomal locus of four mutants showed the presence of an integrase and a transposase in the vicinity of the recombination site (Fig. 2) of the same chromosomal locus. The Km^R cassette in pMKm (Murillo *et al*., 1994) was derived from Tn903 (Oka *et al*., 1981) and contains a partial IS element of 360 bp at each end of the Km^R cassette. In addition, partial DNA fragments of 56 bp and 213 bp of the *epsD* gene were found at the recombination site, suggesting that the unexpected recombination in the SL341E strain probably occurred as a result of the 360-bp inverted repeated IS elements. It is likely that the partial IS elements were recognized by the transposase or an integrase of *R. solanacearum* strain SL341. In fact, genome analysis of *R. solanacearum* strains has revealed an unusual abundance of IS, prophage, and transposable elements (Salanoubat *et al*., 2002; Gabriel *et al*., 2006). Therefore, IS elements flanking the Km^R cassette could serve as a recombination target in *R. solanacearum* strains. Here, we improved the Km^R cassette by removing the IS elements, which will minimize unexpected recombinations during marker exchange mutagenesis and prevent polar mutation, irrespective of insertion orientation of the Km^R cassette.

The Km^R cassette was amplified using two primers, Kan1 (5′-TGTGTCTCAAAATCTCTG-3′) and Kan2 (5′-TTAACCA ATTCTGATTAG-3′). The expected PCR product of 920 bp included the promoter region, but completely excluded the IS element of Tn903. The amplified product was first cloned into pGEM-T Easy vector (Promega, USA). The Km^R cassette was digested with *Eco*RI, Klenow-filled to generate blunt ends, and then transferred into pMKm, which was digested with *Sph*I and Klenow-filled. This cloning process replaced the 1.7-kb Km^R cassette of pMKm with the 920-bp PCR product to generate pMKm2 (Fig. 3).

Here, we also provide pMTp, which can be used to perform



ORF	Coding protein
1	Hypothetical protein
2	Phage intergrase family protein
3	Hypothetical protein
4	TnsA (transposase)

Fig. 2. Genetic organization of the chromosomal locus of *R. solanacearum* SL341E carrying the partial fragment of the marker exchange construct for *epsD* mutation. The ORFs of *R. solanacearum* are indicated in Table. IR denotes an inverted repeat sequence present in the Km^R cassette.

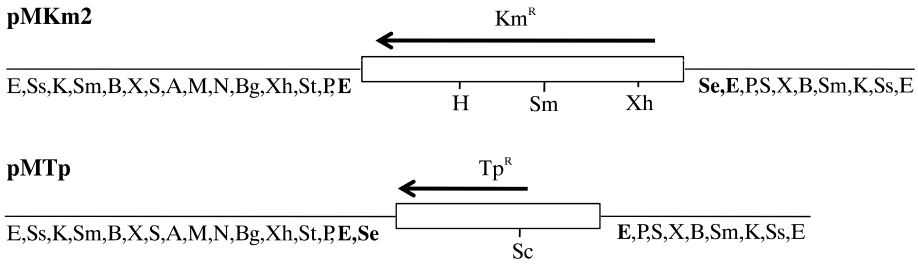


Fig. 3. pMKm2 and pMTp carrying Km^R and Tp^R cassettes, respectively. Restriction enzyme sites: A, *Aat*I; Ac, *Acc*I; Ap, *Apa*I; B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; M, *Mlu*I; N, *Nco*I; Ns, *Nsi*I; P, *Pst*I; S, *Sal*I; Se, *Spe*I; Sm, *Sma*I; Sp, *Sph*I; Ss, *Sst*I; St, *Stu*I; V, *Eco*RV; X, *Xba*I; Xh, *Xho*I. Restriction sites in bold are from pGEM-T Easy vector. Km^R and Tp^R represent resistance to kanamycin and trimethoprim, respectively.

marker exchange mutagenesis using the Tp^R gene from pTn*Mod*-Otp' (Dennis and Zylstra, 1998). The Tp^R gene was amplified by PCR using the primers Trmp-proF (5′-GCCTGTTCGGTT CGTAAACTGTA-3′) and Trmp-R (5′-TTAGGCCACACGTT CAA-3′). The amplified PCR product of 583 bp was first cloned into pGEM-T Easy vector (Promega). The Tp^R cassette was digested with *Eco*RI, Klenow-filled to generate blunt ends, and then transferred into pMKm, which was digested with *Sph*I and Klenow-filled. This cloning process replaced the 1.7-kb Km^R cassette with the 583-bp Tp^R cassette to generate pMTp (Fig. 3). The Tp^R cassette in pMTp could be used as a selectable marker in Gram-negative bacteria with trimethoprim at 100 μg/ml, especially in several *Burkholderia* spp.

Using pMKm2, we performed the desired gene replacement in *R. solanacearum* SL341 to generate nonmucoid mutants. As described above, the *epsD* gene was mutated by inserting the Km^R cassette (digested with *Eco*RI and Klenow-filled) from pMKm2 (Fig. 3) at the internal *Eco*RV site of *epsD*. Two nonmucoid mutants were obtained by conventional marker exchange mutagenesis in the wild-type strain *R. solanacearum* SL341, and the correct gene replacement was verified by Southern hybridization using a DIG-labeled probe of the *epsD* gene (Fig. 4). When 977 bp of the *Sal*I fragment of *epsD* were used as a probe, the expected size difference (920 bp insertion of Km^R cassette) between the wild-type SL341 strain and the two mutant *epsD* loci was observed after *Bam*HI digestion (Fig. 4, lanes 5-7). When the Tp^R cassette in pMTp was used in *B. pyrocinia*, an environmental strain that has been used as a biocontrol agent to control plant diseases and is resistant to several antibiotics, the Tp^R cassette provided a successful selectable marker for performing marker exchange mutagenesis (data not shown).

In conclusion, this study provides the plasmid pMKm2 carrying an improved Km^R cassette, which can be used for efficient marker exchange mutagenesis in many wild-type bacterial strains by preventing polar mutations or aberrant recombinations. The second plasmid pMTp carries a Tp^R cassette, which is useful for marker exchange mutagenesis without polar mutation in many antibiotic-resistant *Burkholderia* spp. and other antibiotic-resistant Gram-negative bacteria.

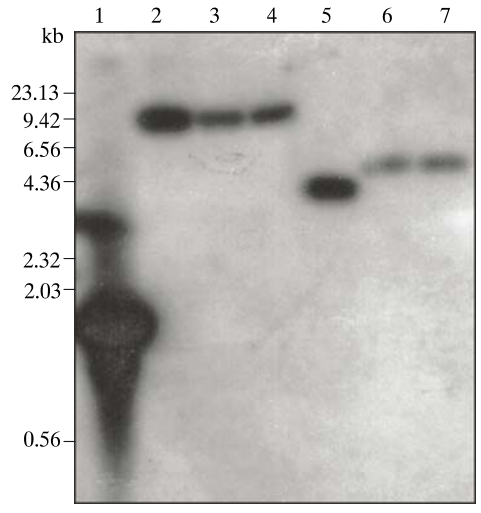


Fig. 4. Southern hybridization with genomic DNA of *R. solanacearum* SL341 and its *epsD* mutants using the kanamycin resistance cassette from pMKm2. Bacterial genomic DNA was digested with *Eco*RI (lanes 2-4) or *Bam*HI (lanes 5-7) for *epsD* gene detection. Hybridization was performed with an *epsD* gene probe. Lanes: 2 and 5, genomic DNA of *R. solanacearum* SL341; 3, 4, 6, 7, genomic DNA of two individual *epsD* mutants of SL341; 1, *epsD* gene cloned in pGEM-T Easy vector and digested with *Eco*RI.

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