RESEARCH NOTE

TN5-OT182 SHOULD NOT BE USED TO IDENTIFY GENES INVOLVED IN BIOFILM FORMATION IN *BURKHOLDERIA PSEUDOMALLEI*

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Abstract. *Burkholderia pseudomallei*, a gram-negative bacterium, is the causative agent of melioidosis. One of the important virulence properties of this bacteria is its ability to form a biofilm. Genes involved in biofilm formation in *B. pseudomallei* have not been thoroughly studied. In this study, Tn5-OT182 mutagenesis was used to isolate of *B. pseudomallei* strain A2 mutants unable to produce biofilm. Ten biofilm-defective transposon mutants were isolated and analyzed. Flanking DNA from each transposon mutant were self-cloned and sequenced, then the sequences were analyzed with the BLAST program. To confirm these genes are involved in biofilm formation, we constructed three gene deletion mutants marked with a tetracycline resistance gene. The constructed *tet*r-marked deletion mutants were checked for correct structure and size by polymerase chain reaction. When subjected to biofilm assay, all tested *tet*r-marked deletion mutants were still able to produce biofilm, indicating the three genes are not involved in biofilm formation. These results suggest integration of Tn5-OT182 in genes not involved in biofilm production can render *B. pseudomallei* unable to produce biofilm by an unknown mechanism. This information demonstrates Tn5-OT182 is not a reliable tool for identifying genes involved in biofilm formation unless a confirmatory experiment is carried out in parallel.

Keywords: *Burkholderia pseudomallei*, melioidosis, biofilm, Tn5-OT182, transposon

INTRODUCTION

Burkholderia pseudomallei is a facultative intracellular gram-negative bacillus that causes melioidosis, a potentially fatal disease in humans and animals. The disease is endemic in Southeast Asia, northeastern Thailand and northern Australia, but sporadic cases have also been reported from other regions of the world (Wiersinga *et al*, 2006). At present, there is no effective vaccine against melioidosis and antibiotic treatment is not fully effective, with relapses despite long courses of drug therapy

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(Cheng and Currie, 2005). *B. pseudomallei* produces several putative virulence factors involved in pathogenesis. These include lipopolysaccharides, a capsule, flagella, pili, a type III secretion system, phospholipase C and several other toxins (Galyov *et al*, 2010). The pathogenetic mechanism of this disease remains to be elucidated in order to understand this complex infection.

B. pseudomallei has been reported to grow in microcolonies and biofilms; *B. pseudomallei* biofilm cells are more resistant to antibiotics (Vorachit *et al*, 1993, 1995), resulting in failure of treatment. The high relapse rate of melioidosis has been hypothesized to be due to reactivation of biofilm cells present in undefined sites. The understanding of genes and factors responsible for biofilm development in this pathogen could lead to better management of melioidiosis. Several genes and one operon involved in biofilm formation by *B. pseudomallei* have been reported. These include the polyphosphate kinase gene (Tunpiboonsak *et al*, 2010) and the *cdpA* (Lee *et al*, 2010) and *rpoE* operons (Korbsrisate *et al*, 2005).

The transposon Tn5-OT182, 10,705 bp in length, is well characterized genetically and has many useful attributes that make it a valuable genetic tool for use with *B. pseudomallei* (Deshazer and Woods, 1999). It integrates randomly into the genome of *B. pseudomallei*. It contains a tetracycline resistance gene which is a useful selective marker in this organism. However, the most useful attribute of this transposon is the ability to self-clone the DNA immediately flanking the site of Tn5-OT182 integration (Merriman and Lamont, 1993). Tn5-OT182 has been used to identify and characterize *B. pseudomallei* mutants defective in motility (DeShazer *et al*, 1997), serum resistance (Deshazer and Woods, 1999), invasion and secretion of exopro-

ducts (Deshazer and Woods, 1999). The present study used Tn5-OT182 to identify and characterize genes that are responsible for biofilm development in *B. pseudomallei*.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

Wild-type *B. pseudomallei* strain A2 isolated from a melioidosis patient at Srinakarind Hospital, Khon Kaen, Thailand was used in this study. *B. pseudomallei* and *Escherichai coli* were cultured in Luria-Bertani (LB) broth or on agar plate at 37ºC. For conjugation method, *B. pseudomallei* and *E. coli* were grown in 2YT broth or on 2YT agar. When appropriate, antibiotics were added at the following final concentrations: tetracycline (Tc), 30 mg/ml; streptomycin (Sm), 300 mg/ml.

Construction of transposon-mediated biofilm mutants of *B. pseudomallei*

In this study, the transposon Tn5-OT182 was used to generate transposon mutants of *B. pseudomallei* strain A2 as previously described (Taweechaisupapong *et al*, 2005). Briefly, *E. coli* SM10 (pOT182) was cultured on 2YT agar containing 30 mg/ml of tetracycline and grown at 37ºC for 18 hours. *B. pseudomallei* A2 was also grown under these conditions but on 2YT agar containing 300 mg/ml of streptomycin. One loopful of the donor strain was mixed with two loopfuls of the recipient strain onto 2YT agar and then the cocultures were incubated at 37ºC for 6 hours. After incubation, the cells were scraped and resuspended in 2 ml of 2YT broth. One hundred and fifty microliters of mating mixture were spread onto 2YT agar containing 30 mg/ml of tetracycline and 300 mg/ml of streptomycin. Tetracycline- and streptomycin-resistant transconjugants were identified after incubation at 37ºC for 24-48 hours.

Table 1
Biofilm mutants of *B. pseudomallei* strain A2 and sites of transposon integration
identified in each biofilm mutant.

Biofilm mutant	Integration site	Location of gene	Putative function
TM1999	BPSS1999	Chromosome 2	Putative transport related, membrane protein or iron permease, FTR1 family
TM0960	BPSS0960	Chromosome 2	Putative Rhs-related membrane protein
TM1993u	Upstream of BPSS1993	Chromosome 2	Not applicable
TM1994u	Upstream of BPSS1994	Chromosome 2	Not applicable
TM2704	BPSL2704	Chromosome 1	Putative OmpW-family exported protein
TM3116	BPSL3116	Chromosome 1	Insertion element hypothetical protein
TM3275	BPSL3275	Chromosome 1	Putative methylase or tetrapyrrole methylase family protein
TM0275	BPSL0275	Chromosome 1	Flagellar basal-body rod protein
TM2589	BPSL2589	Chromosome 1	Putative lipoprotein or TonB domain protein
TM2525	BPSL2525	Chromosome 1	Conserved hypothetical protein or phospholipid N-methyltransferase (PmtA)

Screening for loss of biofilm formation

The Tn5-OT182 mutants of *B. pseudomallei* were screened for loss of biofilm forming ability by a modified microtiter-plate technique as previously described (Taweechaisupapong *et al*, 2005).

Southern hybridization

For Southern hybridization of biofilm-defective transposon mutants, *Eco*RI digests of genomic DNA from *B. pseudomallei* A2 and biofilm mutants were transferred to Biodyne B membrane (Pall Life Scienecs, AnnArbor, MI), and hybridization was performed at 60ºC according to the manufacturer's recommendations. The approximately 4.5 kb *Nde*I/*Xho*I fragment from pOT182 was used as a probe and labeled with digoxigenin (DIG). After hybridization, the membrane was developed by enzyme immunoassay using a DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Diagnostics, Indianapolis, IN).

For Southern hybridization of tetra-cycline resistance gene (*tet*r)-marked gene deletion mutants, genomic DNA from the deletion mutant DM1999, DM3275 and DM2589 were digested with *Cla*I, *Nco*I/*Apa*LI, and *Blp*I, respectively. The transferred membranes were hybridized with *tetA* gene probe at 50ºC and the membrane was developed as previously described (Taweechaisupapong *et al*, 2005).

RESULTS

A total of 3,300 transposon mutants were screened for loss of biofilm formation by a modified microtiter-plate test (Stepanovic *et al*, 2000) and 10 stable biofilm-defective transposon mutants were isolated. On Southern blot analysis, each of the ten biofilm-defective transposon mutants contained a single copy of Tn5-OT182 integrated at different locations. The DNA immediately flanking the site of integration in each biofilm mutant was self-cloned and sequenced; each sequence

was analyzed with BLASTN and BLASTX databases (Table 1).

Transposon is known to have polar effects on downstream genes. Therefore, a gene integrated by Tn5-OT182 shows a different phenotype when this gene is disrupted by a different marker, such as an antibiotic resistance gene. To confirm the genes integrated by Tn5-OT182 in biofilm-defective mutants were involved in biofilm formation, we constructed three tetracycline resistance gene (*tet^r*)-marked gene deletion mutants with three selected genes containing integration sites inside the gene. Briefly, BPSS1999, BPSL3275, and BPSL2589 were amplified by polymerase chain reaction; each with two sets of primers designed to generate an internal deletion of each gene. The two amplified fragments were digested, ligated and cloned into pDM4, a suicide vector (Milton *et al*, 1996). Then, a *tet^r* cassette was cloned into a unique site inside each mutated gene and the correct recombinant plasmid containing the *tet^r*-marked gene was introduced into *B. pseudomallei* strain A2 and strain K96243 (K9 for short) by conjugation. Three correct *tet^r*-marked gene deletion mutants (DM) from strain A2 (designated DM1999A2, DM3275A2 and DM2589A2) and three deletion mutants from strain K9 (designated DM1999K9, DM3275K9 and DM2589K9), confirmed by PCR, DNA sequencing and Southern hybridization, were isolated. Each mutant represented a deletion mutant derivative of BPSS1999, BPSL3275, and BPSL2589, respectively. Surprisingly, when assessed for biofilm formation, all six deletion mutants were still able to form biofilm comparable to those seen for the wild types (Fig 1).

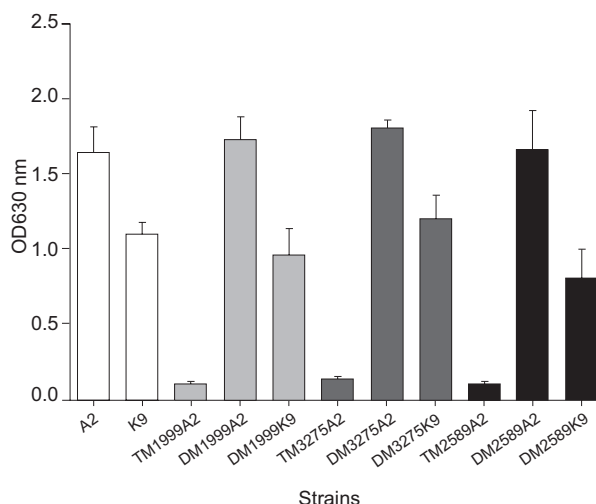## DISCUSSION

Tn5-OT182 has been used to identify

Fig 1–Biofilm formation by *B. pseudomallei* wild types (A2 and K9), three Tn5-OT182 mutants (TM) and three deletion mutants (DM) of A2 and K9 as measured by a modified microtiter-plate test. Crystal violet was used to quantitate biofilm formation as previously described (Stepanovic *et al*, 2000). The bars indicate means ± standard error of the three experiments.

genes involved in biofilm development in *B. pseudomallei* strain H777 (Taweechaisupapong *et al*, 2005). Both mutants (M6 and M10) are still virulent in a mouse model leading to the conclusion that virulence of *B. pseudomallei* does not correlate with biofilm formation. However, the authors have not confirmed that both genes (in M6 and M10) integrated by the transposon are involved in biofilm formation. The results from the present study indicate that the gene integrated by Tn5-OT182 may not be involved in biofilm formation as suggested by the phenotype of the corresponding biofilm-defective transposon mutant. The results also suggest that integration of Tn5-OT182 in genes not involved in biofilm production can render *B. pseudomallei* unable to produce biofilm by unknown mechanisms. We could not confirm the

other genes because they were either too big or the integration site was outside the gene, as found in biofilm mutants TM1993u and TM1994u (Table 1). These data demonstrate that Tn5-OT182 is not a reliable tool for use to identify genes involved in biofilm formation unless a confirmation experiment is carried out in parallel.

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