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Construction of a Large-Scale *Burkholderia cenocepacia* J2315 Transposon Mutant Library

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Abstract. *Burkholderia cenocepacia*, a pathogenic member of the *Burkholderia cepacia* complex (Bcc), has emerged as a significant threat towards cystic fibrosis patients, where infection often leads to the fatal clinical manifestation known as cepacia syndrome. Many studies have investigated the pathogenicity of *B. cenocepacia* as well as its ability to become highly resistant towards many of the antibiotics currently in use. In addition, studies have also been undertaken to understand the pathogen's capacity to adapt and survive in a broad range of environments. Transposon based mutagenesis has been widely used in creating insertional knock-out mutants and coupled with recent advances in sequencing technology, robust tools to study gene function in a genome-wide manner have been developed based on the assembly of saturated transposon mutant libraries. In this study, we describe the construction of a large-scale library of *B. cenocepacia* transposon mutants. To create transposon mutants of *B. cenocepacia* strain J2315, electrocompetent bacteria were electrotransformed with the EZ-Tn5 <TET-1> transposome. Tetracyline resistant colonies were harvested off selective agar and pooled. Mutants were generated in multiple batches with each batch consisting of ~ 20,000 to 40,000 mutants. Transposon insertion was validated by PCR amplification of the transposon region. In conclusion, a saturated *B. cenocepacia* J2315 transposon mutant library with an estimated total number of 500,000 mutants was successfully constructed. This mutant library can now be further exploited as a genetic tool to assess the function of every gene in the genome, facilitating the discovery of genes important for bacterial survival and adaptation, as well as virulence.

Keywords: *Burkholderia cenocepacia*, *Burkholderia cepacia* complex, transposon, mutant.
PACS: 87.19.xb, 87.80.St

INTRODUCTION

Burkholderia cepacia complex, or Bcc, is a group of gram negative bacteria comprising of at least 17 members, all of which are found in diverse environmental niches. Although a few Bcc species have the potential to act as bioremediation and biocontrol agents, a number of them are infectious towards plants, animals and even humans. These Bcc species have emerged as a significant threat towards cystic fibrosis patients as they are able to exist as opportunistic pathogens and cause fatal pneumonia or clinical manifestations known as cepacia syndrome, resulting in early death [1-2]. *B. cenocepacia* is a member of the Bcc that is strongly associated with infection in a majority of Bcc infected CF patients. Although not as common *Pseudomonas aeruginosa*, another major pathogen which causes respiratory infection in CF patients, the high level of clinical antibiotics resistance demonstrated by Bcc has further limited the effective treatments that can be used to eradicate Bcc infections [3].

Over the past decades, transposon-based mutagenesis has been widely used as a powerful tool in functional genomics studies. Tn-based mutagenesis is used to create insertional knockout mutants which are often used for the characterization of gene functions and essentiality. The ability of a transposon to move from one site to another site of the genome through the event of transposition facilitates the process of gene manipulation where gene disruption can occur through targeted mutagenesis or by random transposon insertion [4-5]. Many studies have been done using loss-of-function transposon mutants and several Bcc virulence factors have been identified through mutant screening, including biofilm formation related proteins and toxins [2]. Recent advances in high-throughput technologies such as next-generation sequencing allow traditional transposon mutagenesis systems to be further developed as a robust tool to study gene functionality in a genome-wide manner, where the assessment of gene function can be done simultaneously based on the assembly of saturated transposon mutant libraries [6]. Due to the current lack of understanding of the pathogenicity of Bcc, in this study, we aimed to create a large-scale Bcc transposon mutant library. This mutant library can be further exploited as a genetic tool to assess every gene in the

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bacterial genome, facilitating the discovery of genes important for bacterial survival and adaptation as well as virulence.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions

The bacteria strain used in this study is *Burkholderia cenocepacia* J2315 (LMG16656, BCCM/LMG Bacteria Collection, Ghent, Belgium). The wild type strain was routinely grown on Ashdown agar and Brain Heart Infusion (BHI) broth. To select transposon mutants, modified LB agar (30 g of tryptone, 10 g of yeast extract and 18 g of bacteriological agar) supplemented with tetracycline (500 µg/mL) was used as the selective media.

Preparation of Electrocompetent Cells

BccJ2315 bacterial cells were made electrocompetent using the method reported by Dubarry et al. [7] with minor modifications. Briefly, BccJ2315 was grown in 50 mL of SOB medium supplemented with 0.8% glycine at 20°C with shaking (150 rpm or less) until an OD_{595nm} of 0.08 was achieved. Once achieved, the bacteria were chilled on ice for 30 min and centrifuged at 6,000 × g for 10 min at 4°C. The harvested cells were then gently washed with pre-chilled 10% glycerol and centrifuged as above. The washing step was repeated three times and finally the cells were resuspended in 200 µL of 10% glycerol. Electrocompetent cells were used immediately for electroporation.

Electrotransformation of BccJ2315

To construct the transposon mutants, the EZ-Tn5 <TET-1> Insertion Kit from Epicentre Illumina was used (FIGURE 1). Transposome was prepared following the manufacturer's instruction and kept at -20°C until use. One µL of tranpsosome was added to 50 µL of electrocompetent cells and left to stand stand on ice for 5 min before the cell suspension was transferred to an ice-cooled 2-mm gap electroporation cuvette. Electroporation was performed at a pulse of 2.5 kV and 200 uF (Gene Pulser Xcell, Bio-Rad). SOC medium (950 µL) was immediately added and electroporated cell suspension was incubated at 37°C with aeration for 2 hours. Cells were then spread on modified LB agar supplemented with tetracycline (500 µg/mL) and incubated at 37°C for 24 hours. Estimation of the number of positive transformants or mutants obtained was done by calculating the colony forming units (CFU) in a portion of each plate.

Validation of Transposon Insertion

To detect the presence of transposon insertion in the positive colonies, six random colonies were picked individually and genomic DNA for each was extracted using the MasterPure DNA Purification Kit (Epicentre, Illumina). PCR was performed with primers Tnp_FP (5' TTGGCATGGATTGTAGGCG 3') and Tnp_RP (5' CAACCTGAAGCTTGCATGCC 3') using the follow program: initial denaturation at 94°C for 2 minutes, 30 cycles of 94°C for 30 seconds, 52°C for 45 seconds, 72°C for 1 minute and final elongation at 72°C for 5 minutes. Wild type BccJ2315 genomic DNA was included as a negative control.

Pooling of Transposon Mutants

Electrotransformations were performed in large scale using the method as described above. Mutants were washed with BHI broth, harvested off from plates using a cell scrapper and stored in 30% glycerol at -80°C. Electrotransformations were repeated in multiple batches until an estimated total number of 500,000 mutants were reached. Batches of mutants were then pooled to create a saturated transposon mutant library.

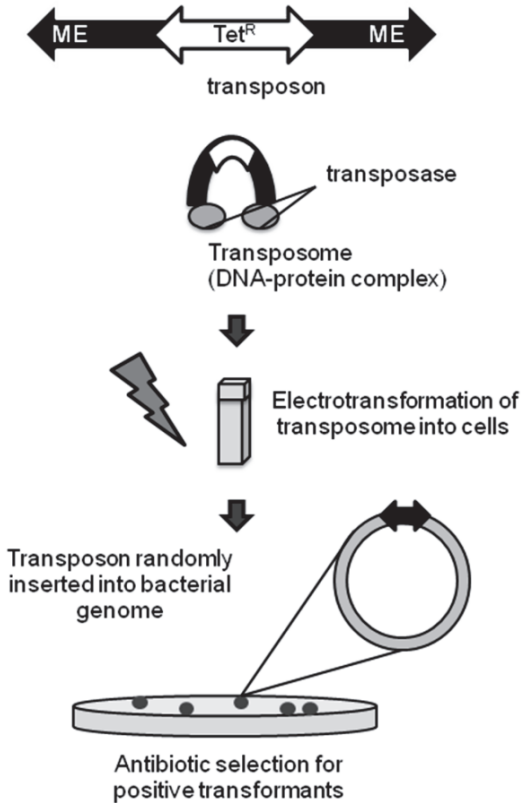

FIGURE 1. Schematic diagram describing the construction of transposon mutants. Tn5 transposase and Tn5 transposon formed a stable DNA-protein complex (transposome) in the absence of Mg^{2+}. Transposome was introduced into bacterial cells through electroporation. The presence of Mg^{2+} in the cellular environment allowed transposition to occur and the transposon was randomly inserted into the bacterial genome. Positive transformants were selected on tetracycline containing media.

RESULTS AND DISCUSSION

The standard electrotransformation protocol routinely used for *E. coli* was found to be inefficient for BccJ2315, as no transformants are generally produced [7]. In this study, we adapted the BccJ2315 electrotransformation protocol established by Dubarry et al. and obtained a large number of mutants generated per µL of transposome used. The improved method of electrocompetent cells preparation by growing bacterial cells under ambient temperature instead of 37°C and also to a reduced OD_{595nm} (0.04 - 0.08) before harvesting was found to increase the electrotransformation efficiency of BccJ2315. Furthermore, as reported, the addition of glycine into SOB medium also increased the transformation efficiency most likely due to the ability of glycine to weaken the bacterial cell wall as observed for Gram-positive bacteria [7]. On average, approximately 1,500 mutants were created for every microliter of transposome used.

To confirm that the positive colonies obtained from the electrotransformation are transposon-inserted mutants and not the wild type strain, we performed PCR using primers designed to amplify the transposon region. A product size of 312 bp is expected if the transposon is present in the bacterial genome. Of all the six randomly selected tranformants, we observed a single PCR band of the expected size indicating the presence of transposon in the genomic DNA (FIGURE 2). Moreover, no amplified product was observed for wild type J2315 DNA, further confirming that the transposition event only occurred in the mutants.

Once the insertion of transposon was validated, large-scale mutant construction was carried out to generate as many mutants as possible until a degree of saturation was achieved. Using the optimized electrotransformation protocol described above, BccJ2315 transposon mutants were generated in multiple batches. About 20 or more electotransformations were performed for each batch, allowing an estimated 20,000 to 40,000 transposon mutants created per batch. With more than 10 batches, approximately 500,000 BccJ2315 transposon mutants were

successfully generated. These transposon mutants were pooled to create a saturated BccJ2315 transposon mutant library.

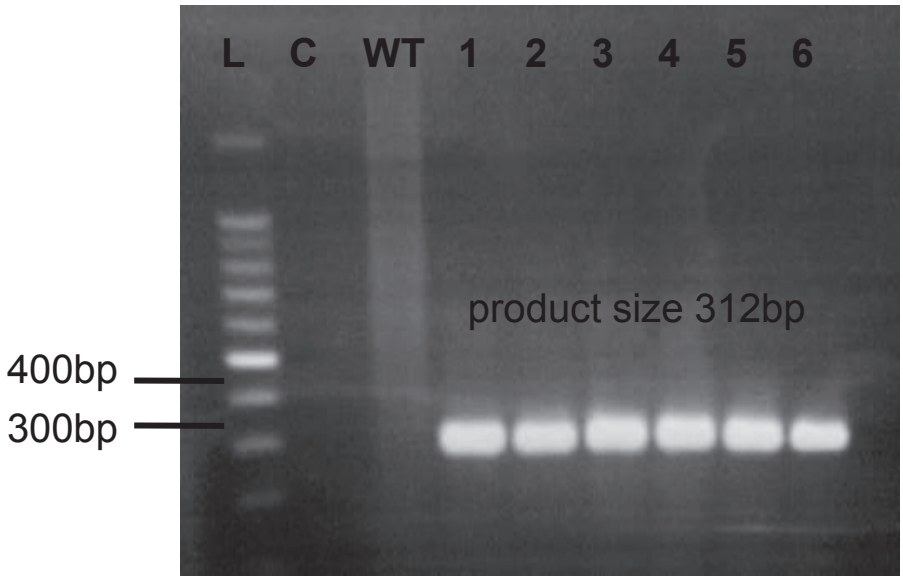

FIGURE 2. Validation of transposon insertion in positive transformants. L: 100 bp DNA ladder (Promega); C: primers only, no DNA template; WT: BccJ2315 wild type genomic DNA; Lane 1 - 6: Genomic DNA from randomly picked colonies (BccJ2315 mutants). The expected product size is 312 bp.

In summary, we have successfully constructed a large-scale BccJ2315 transposon mutant library consisting of ~500,000 mutants. In tandem with recent advanced approaches, this mutant library can now be used as a tool to facilitate gene functionality studies of this pathogen in a genome wide manner, contributing not only to the discovery of genes important for the bacterial survival and adaptation, but also to understanding the molecular mechanisms that underlie the pathogenicity of BccJ2315.

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