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Commentary Open Access

Simple Method for Markerless Gene Deletion in Multidrug Resistant *Acinetobacter baumannii*

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Commentary

Acinetobacter baumannii is an important, opportunistic, multidrug resistant human pathogen causing nosocomial infections in both community and hospital settings worldwide [1]. The importance of this emerging pathogen is further fortified by the fact that over 200 different isolates have already been sequenced [2-5]. Over the past few years, the identification of wide number of genes in *A. baumannii* has led the researchers to develop new tools for the construction of deletion mutants in order to study the functional aspects of various genes involved in the fitness of the organism and in virulence. The usage of a double cross over method along with cloning based on overlap extension polymerase chain reaction (PCR) has been widely used for the construction of deletion mutants, where a plasmid harboring the mutated allele of the gene to be deleted is integrated into the target sequence of *A. baumannii* for the allelic exchange to occur [6]. However, the above method is time-consuming and inefficient, as it demands extensive screening for desired chimeric plasmid after cloning because of the possibility of high background of false positive colonies. In addition, the method is limited by the availability of suitable restriction enzymes and antibiotic resistance cassette in the cloning step. Recently, a system that utilizes RecET recombinase and flippase (FLP)/ FLP recognition target (FRT) systems was reported for markerless gene deletion in *A. baumannii*. However, the absolute requirement of two antibiotic resistance markers makes the system less suitable for the construction of deletion mutants of multidrug resistant clinical isolates of *A. baumannii* as the availability of suitable antibiotic resistance markers are highly limited.

Our laboratory is focusing on the identification and functional characterization of genes involved in pathogenesis, biofilm formation and antibiotic resistance, and we were keen on developing new strategies to effectively construct deletion mutants of *A. baumannii*. In our new approach, a blunt-ended PCR product containing upstream and downstream of the target gene, and an antibiotic cassette is amplified by overlap extension PCR and cloned into a suicide vector, pHKD01 [7]. The recombinant plasmid is then integrated into the target sequence of *A. baumannii* chromosome using conjugation followed by homologous recombination. The single cross over mutants are selected on the medium supplemented with appropriate antibiotic. A second homologous recombination by sucrose counter-selection results in allelic exchange by which the antibiotic resistance cassette with the plasmid backbone is eliminated. To validate the new method, the genes, *bas*D and *bau*A which are required for iron acquisition of *A. baumannii* [8] was successfully deleted. *bas*D and *bau*A mutants were not able to grow under iron-depleted conditions as these genes are essential for the survival of *A. baumannii* under iron-depleted conditions [8]. The method was effectively applied to construct double deletion mutant of *A. baumanni* by deleting *cir*A gene in *bau*A deletion mutant. In addition, the strategy was feasible in constructing deletion mutants in different *A. baumannii* clinical strains. The average success rate in constructing deletion mutant using this tool in *A. baumannii* was 48%.

The incorporation of antibiotic cassette downstream of the target gene by overlap extension PCR eliminates background of false positive colonies in the cloning step, thereby making the above method a less time-consuming one. The vector, pHKD01 that was constructed for the cloning purpose carries alternative cloning sites for direct cloning of blunt-ended PCR products. Thus, the availability of restriction enzymes is not limited anymore in cloning. In addition, the antibiotic resistance cassette is easily excised by the second cross over resulting in markerless gene deletion. Hence, the same antibiotic cassette can be recycled when multiple genes are targeted for modification in the same bacterial cell. Moreover, the method does not demand the construction of new vectors containing suitable antibiotic resistance markers for the construction of deletion mutants of multidrug resistant clinical isolates. The applicability of this method in the construction of single or double deletion mutants of clinical isolates of multidrug resistant *A. baumannii* provides us a better chance in studying various genes which are involved in virulence, biofilm formation, antibiotic resistance and multidrug efflux pumps [7] (data not published). Although not tested, it is likely that this novel method may also work with other pathogenic bacteria, in which the genetic manipulation techniques are generally less well established.

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