

Identification of a Potential Virulence-Related Operon by Rescue Cloning EZ::TN™ Transposon Insertion Sites from a Bacterial Pathogen

Caleb W. Dorsey, Andrew P. Tomaras, John C. Berschback, and Luis A. Actis
Miami University, Oxford, Ohio

Introduction

Acinetobacter baumannii is a Gram-negative, opportunistic pathogen that causes severe infections in compromised patients. Since this pathogen resists a wide range of antimicrobial compounds these infections are a serious concern in human medicine. However, little is known about the genes and factors involved in *Acinetobacter* basic physiology and virulence properties. Here we demonstrate that electroporation of the EZ::TN™ <R6K γ ori/KAN-2>Tnp Transposome™ proved to be an effective and simple approach to generate random transposon insertions in the *A. baumannii* 19606 genome. Moreover, we were able to recover or “rescue” the interrupted chromosomal regions as plasmids due to the presence of an origin of replication within the transposable element.

Materials and Methods

Insertional mutagenesis, mutant screening, and rescue of interrupted sequences

The strategy used to generate and screen *A. baumannii* 19606 insertion mutants is shown in Figure 1. The EZ::TN <R6K γ ori/KAN-2>Tnp Transposome was electroporated into *A. baumannii* 19606 electrocompetent cells with the 2510 Eppendorf electroporator and 2-mm-wide cuvettes at 2.5 kV. Electroporated cells were suspended

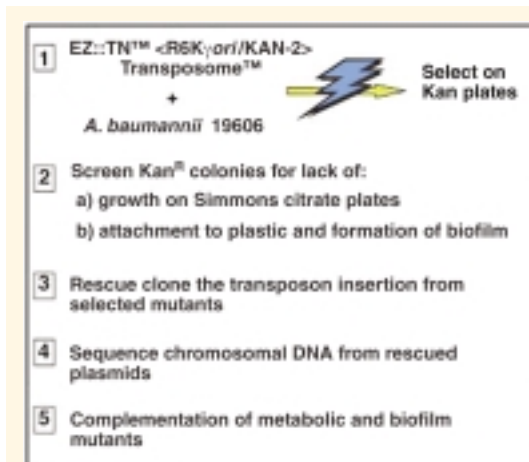


Figure 1. Overview of the strategy used to generate and screen EZ::TN™ <R6K γ ori/KAN-2> Transposon insertions in *A. baumannii* 19606. The EZ::TN™ Transposome™ is the stable complex formed between an EZ::TN Transposon and EZ::TN™ Transposase that can be electroporated into bacterial cells. Once inside the cell, the transposon component is randomly inserted into the host's genomic DNA.

immediately in SOC broth, allowed to recover at 37°C for 1 hour, and then plated on LB agar containing 40 μ g/ml kanamycin. Growth on Simmons citrate agar and attachment to plastic tubes or microtiter plates were used to identify insertion derivatives affected in metabolic and attachment/biofilm functions, respectively.

Genomic regions harboring EZ::TN™ <R6K γ ori/KAN-2> Transposon insertions were rescued as shown in Figure 2. The genomic DNA flanking an insertion was sequenced bidirectionally with primers (supplied with the kit) that anneal near the ends of the transposon. Further extension of nucleotide sequences was done

using custom-designed primers and plasmid DNA as a template. Sequencher 4.1.2 (Gene Codes Corp.) was used to examine and assemble nucleotide sequences, which were analyzed with DNASTAR, BLAST, and the software available through the ExpASY Molecular Biology Server (<http://www.expasy.ch>).

Biochemical and genetic complementations

Positive growth on supplemented Simmons citrate agar was recorded as concomitant detection of abundant bacterial growth on the streaked areas and change of color from green to blue in the surrounding areas, after overnight incubation at 37°C. Genetic complementation of insertional mutants required PCR amplification of the uninterrupted gene of interest from the genome of the parental strain and cloning in the shuttle vector pWH1266.¹ Restoration of cell attachment/biofilm formation was determined after 24 hours stagnant incubation of LB broth cultures at 37°C in polystyrene tubes.

Results and Discussion

Electroporation with 1 μ l of the EZ::TN <R6K γ ori/KAN-2>Tnp Transposome generated ~3,500 Kan^R colonies. Southern blot analysis of restricted genomic DNA isolated from 18 of these Kan^R derivatives showed that all of them harbored the kanamycin-resistance gene, which could not be detected in the genome of the parental strain (data not shown). With the exception of one, all the derivatives appeared to contain a single EZ::TN <R6K γ ori/KAN-2> Transposon insertion.

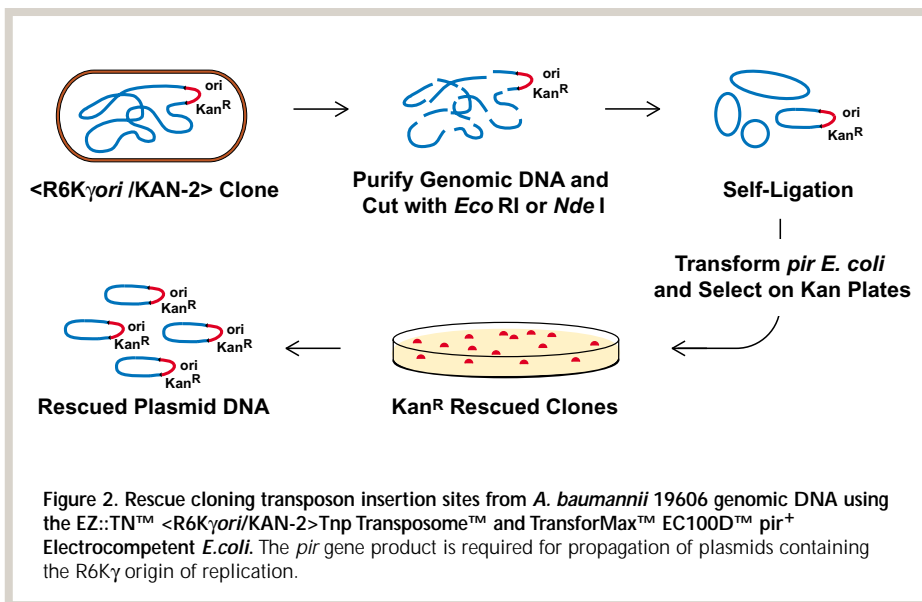


Figure 2. Rescue cloning transposon insertion sites from *A. baumannii* 19606 genomic DNA using the EZ::TN™ <R6K γ ori/KAN-2>Tnp Transposome™ and TransforMax™ EC100D™ pir⁺ Electrocompetent *E. coli*. The *pir* gene product is required for propagation of plasmids containing the R6K γ origin of replication.

The efficient rescue of the interrupted chromosomal sequences as plasmids allowed us to rapidly determine the precise EZ::TN <R6K γ ori/KAN-2> Transposon insertion site in selected mutants. Sequence analysis demonstrated that mutants unable to grow on Simon citrate agar were impaired in functions required for the generation of energy or the production of precursors required for the biosynthesis of nucleic acids, proteins, and cell wall components (Table 1). For example, mutants 9, 19, and 23 carry EZ::TN <R6K γ ori/KAN-2> Transposon insertions within genes required for tryptophan biosynthesis. Growth of these mutants was restored to levels similar to those of the parental strain when this chemically defined medium was supplemented with 1 mM tryptophan. In contrast, these three insertion derivatives and the parental strain showed identical growth curves when cultured in LB broth.

The attachment/biofilms assays yielded mutant 144, which has an EZ::TN <R6K γ ori/KAN-2> Transposon inserted within a gene encoding a polypeptide highly similar to the *Vibrio parahaemolyticus* CsuE protein. The *csuE* gene is the last component of *csuABCDE*, a predicted polycistronic locus that potentially codes for functions required for the secretion and assembly of bacterial

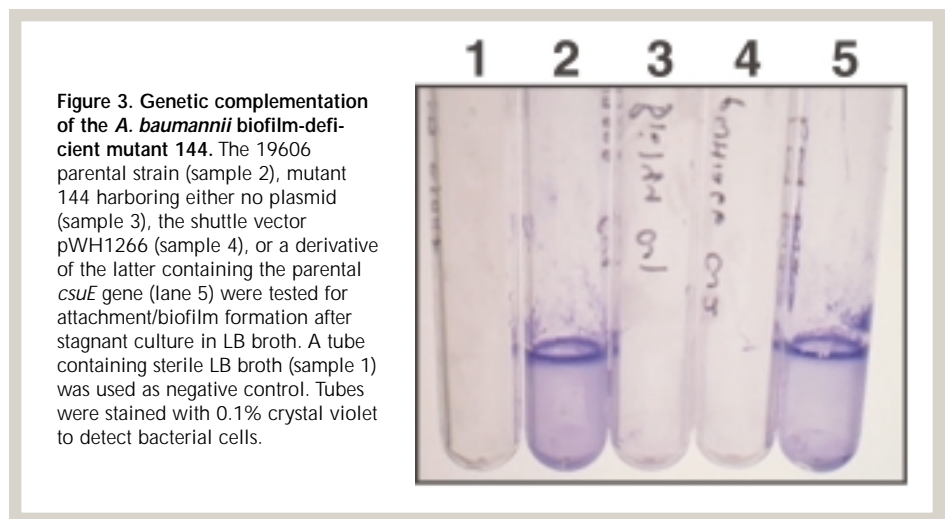


Figure 3. Genetic complementation of the *A. baumannii* biofilm-deficient mutant 144. The 19606 parental strain (sample 2), mutant 144 harboring either no plasmid (sample 3), the shuttle vector pWH1266 (sample 4), or a derivative of the latter containing the parental *csuE* gene (lane 5) were tested for attachment/biofilm formation after stagnant culture in LB broth. A tube containing sterile LB broth (sample 1) was used as negative control. Tubes were stained with 0.1% crystal violet to detect bacterial cells.

proteins involved in pili formation in other Gram-negative bacteria.² Previous work has established that this type of cell surface appendage is involved in cell attachment and biofilm formation on abiotic surfaces. Electroporation of a pWH1266 derivative harboring the interrupted *csuE* gene restored the ability of mutant 144 to attach to and form biofilm on plastic surfaces in a fashion similar to the parental strain (Figure 3). Currently, we are determining the nucleotide sequence and genetic structure of the *A. baumannii* 19606 *csu*-like operon,

whose products have the potential of playing an important role in the biology and virulence properties of this bacterium.

In summary, the utilization of the EZ::TN <R6K γ ori/KAN-2>Tnp Transposome system proved to be an efficient tool for the generation of random *A. baumannii* mutants affected in metabolic and attachment/biofilm functions. This convenient approach should facilitate the genetic and functional analysis of this poorly characterized opportunistic human pathogen.

References

1. Hunger, M. *et al.* (1990) *Gene* **87**, 45.
2. Thanassi, D.G. *et al.* (1998) *Curr. Opin. Microbiol.* **1**, 223.

Table 1. Characterization of EZ::TN™ <R6K γ ori/KAN-2> Transposon insertions in *A. baumannii* 19606.

Mutant	Insertion Site ^a	Gene - Function Disrupted ^b
1	GCCCTAAAA	<i>dapA</i> – dihydrodipicolinate synthase
9	CGCGGATAC	<i>trpD</i> – anthranilate phosphoribosyltransferase
10	GTTTATTCA	<i>argF</i> – ornithine carbamoyltransferase
12	GGGCCATAC	<i>argG</i> – argininosuccinate synthase
13	ATAGAATGG	<i>aceE/aceA</i> – pyruvate dehydrogenase E1
15	AATGGAAAC	<i>proA</i> – g-glutamyl phosphate reductase
16	AATATACGT	<i>cysII/nirA/sir</i> – sulfite reductase
19	CTACGATGC	<i>trpE</i> – anthranilate synthase
23	CTATTCACA	<i>trpE</i> – anthranilate synthase
26	CATGTGAAT	<i>cysII/nirA/sir</i> – sulfite reductase
28	CATGTGAAT	<i>cysII/nirA/sir</i> – sulfite reductase
31	CTGCAAACC	<i>hisH</i> – glutamine amidotransferase
44	GTTTTACGT	<i>cysII/nirA/sir</i> – sulfite reductase
144	GTCACAAAC	<i>csuE</i> – chaperone/usher secretion system

^aBases indicate a 9-bp duplication that flanks each side of the inserted transposon.

^bPotential genes and functions were predicted by BLASTp and BLASTx searches.

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EZ::TN™ <R6K γ ori /KAN-2>Tnp Transposome™ Kit

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TransforMax™ EC100D™ *pir*⁺ Electrocompetent *E. coli*

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Maintains clones at 15 copies per cell.
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EC6P095H 5 X 100 μ l
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Includes control vector containing an R6K γ ori.