Genetic tools for manipulating \textit{Acinetobacter baumannii} genome: an overview

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\textit{Acinetobacter baumannii} is an emerging nosocomial pathogen involved in a variety of infections ranging from minor soft-tissue infections to more severe infections such as ventilator-associated pneumonia and bacteremia. \textit{A. baumannii} has become resistant to most of the commonly used antibiotics and multidrug-resistant isolates are becoming a severe problem in the healthcare setting. In the past few years, whole-genome sequences of $>$200 \textit{A. baumannii} isolates have been generated. Several methods and molecular tools have been used for genetic manipulation of various \textit{Acinetobacter} spp. Here, we review recent developments of various genetic tools used for modification of the \textit{A. baumannii} genome, including various ways to inactivate gene function, chromosomal integration and transposon mutagenesis.

**Introduction**

The genus \textit{Acinetobacter} belongs to the family \textit{Moraxellaceae} within the order \textit{Gammaproteobacteria} and is composed of a taxonomically highly heterogeneous group of bacteria. Bacteria belonging to \textit{Acinetobacter} are ubiquitous in the environment and are predominantly present in the soil. At present, the genus comprises $\sim$35 validly named species as listed at \textit{Bacterionet} (http://www.bacterionet.org/). This list is growing at an increasingly high rate; within the last 2 years alone, six species were validated. The genus also consists of a few unnamed genomic species defined by nucleic acid hybridization and numerous isolates with uncertain taxonomic status (Espinal et al., 2012; Visca et al., 2011). \textit{Acinetobacter baumannii} is clinically the most relevant species that has been frequently associated with nosocomial infections and outbreaks. \textit{A. baumannii} can cause a variety of infections ranging from superficial skin and soft-tissue infections to more severe diseases such as bacteraemia and pneumonia (McConnell et al., 2013; Peleg et al., 2008a; Roca et al., 2012). Hospital-acquired pneumonia is the most common clinical infection caused by \textit{A. baumannii} where the mortality rate is high. Community-acquired pneumonia caused by \textit{A. baumannii} is much less frequent than nosocomial infections, but the mortality rate is similar to hospital-acquired pneumonia. \textit{A. baumannii} is also responsible for bloodstream infections where the mortality rates are also high. Some other infections caused by \textit{A. baumannii} are burn and wound infections, urinary tract infections, meningitis, osteomyelitis, and endocarditis associated with prosthetic valves (Howard et al., 2012; McConnell et al., 2013; Roca et al., 2012). The primary reason that \textit{A. baumannii} is successful as a human pathogen is due to its multidrug resistance, which is generally assimilated by acquisition of various genetic elements such as plasmids, insertion sequences and resistance islands (Dijkshoorn et al., 2007; Gordon & Wareham, 2010; Imperi et al., 2011). The other features that render this pathogen successful are its ability to resist desiccation, which allows the pathogen to persist on abiotic surfaces present in healthcare settings, and to colonize asymptotically within the human host, enabling its rapid spread (Fournier et al., 2006; Jawad et al., 1998; Marchaim et al., 2007; Wendt et al., 1997).

Biofilm formation on abiotic surfaces has been suggested to play a role in \textit{A. baumannii} persistence and is a debatable issue (Antunes et al., 2011; Kaliterna & Goic-Barisic, 2013; Longo et al., 2014; McQueary & Actis, 2011; Rodriguez-Bano et al., 2008). \textit{A. baumannii} expresses various proteins, extracellular polysaccharide and type IV pili, all of which are necessary for biofilm formation (Gayoso et al., 2014; Goh et al., 2013; Shin et al., 2009). Although \textit{A. baumannii} has been described as non-motile, this organism displays twitching motility and swarming-like motility that are also mediated by type IV pili (Clemmer et al., 2011; Eijkelkamp et al., 2011b; Harding et al., 2013; Skiebe et al., 2012). Furthermore, \textit{A. baumannii} and other \textit{Acinetobacter} spp. are naturally competent, which enables them to take up genetic elements encoding antibiotic resistance from the environment (Harding et al., 2013; Ramirez et al., 2010; Wilharm et al., 2013). Type IV pili also play a significant role in DNA uptake during competence. In addition to type IV pili, a few other virulence factors have been studied to some extent, but the role of the vast majority of the virulence factors in pathogenesis remains poorly understood (Bahl et al., 2014; Eijkelkamp et al., 2014; Elhosseiny et al., 2014).

**Abbreviations:** Flp, flippase; FRT, flippase recognition target; MDR, multidrug-resistant; TraSH, transposon site hybridization.
There is no doubt that \textit{A. baumannii} is an important emerging pathogen. The importance is further strengthened by the fact that >200 different isolates have already been sequenced including several multidrug-resistant (MDR) strains (Adams \textit{et al.}, 2008; Chen \textit{et al.}, 2011; Gao \textit{et al.}, 2011; Iacono \textit{et al.}, 2008). The availability of a large number of genome sequences of \textit{A. baumannii} offers a great advantage in understanding the role of various genetic factors involved in the fitness of the organism and in virulence. However, genetic analysis of \textit{A. baumannii} has thus far been limited due to a lack of advanced genetic tools.

In general, the function of a gene is assessed by either inactivation or selective modification. In the case of \textit{A. baumannii}, the classical strategy for obtaining gene deletion variants is based on either single-crossover insertion duplication mutagenesis (Aranda \textit{et al.}, 2010; Clemmer \textit{et al.}, 2011; Heritier \textit{et al.}, 2005) or allelic exchange by double-crossover recombination (Amin \textit{et al.}, 2013; Aranda \textit{et al.}, 2010; Cerqueira \textit{et al.}, 2014; Harding \textit{et al.}, 2013).

In addition to these approaches, various transposon mutagenesis strategies are employed for obtaining gene deletion variants (Clemmer \textit{et al.}, 2011; Dorsey \textit{et al.}, 2002; Jacobs \textit{et al.}, 2010, 2014c; Smith \textit{et al.}, 2007; Umland \textit{et al.}, 2012; Wang \textit{et al.}, 2014). Recently, Jacobs \textit{et al.} (2014b) published a short laboratory manual on the practical aspects of genetic manipulation of \textit{A. baumannii}. Whilst their publication is very useful, it does not cover all of the aspects of gene manipulation. In this review, we briefly elucidate most of the methods currently available for the manipulation of the \textit{A. baumannii} genome. We also examine various transposon mutagenesis strategies for the manipulation of the \textit{A. baumannii} genome.

**Methods for gene transfer in \textit{A. baumannii}**

Although naturally competent, the most commonly used method for gene transfer is through electroporation. Electroporation relies on the use of electric pulses to transiently produce pores in the bacterial membrane (Miller \textit{et al.}, 1988). These pores are large enough to allow the passage of DNA, protein and DNA–protein complexes from the outside milieu into the cells. The efficiency of electroporation depends on the capacity of the membrane to reassemble, which allows the cell to survive the electric shock (Drury, 1996; Miller, 1994). The two main parameters that dictate the success of electroporation are the strength and the duration of the electric pulse. For prokaryotic electroporation, the buffer containing the cells and DNA should be free of electrolytes and salts so that the solution can provide high resistance (Drury, 1996; Miller, 1994). Lowering the volume also increases the resistance. Electroporation is highly efficient in \textit{A. baumannii} and one can obtain ~$10^8$ transformants per microgram of replicative circular plasmid DNA. However, the efficiency depends on the size of the plasmids; smaller plasmids tend to yield higher efficiency than larger plasmids (Wirth \textit{et al.}, 1989). Linear DNA can also be used for efficient gene transfer by electroporation in \textit{A. baumannii}; although the efficiency is somewhat low (<$10^2$ transformants $\mu$g$^{-1}$). Linear DNA transformation is generally used for direct gene replacement events (see below). As in \textit{Escherichia coli}, \textit{A. baumannii} also encodes the RecBCD exonuclease that generally degrades the unprotected linear DNA (de Vries & Wackernagel, 2002; Kickstein \textit{et al.}, 2007). It appears that electroporation transiently inactivates the exonucleolytic function of RecBCD in \textit{E. coli}, thus allowing gene replacement to occur (El Karoui \textit{et al.}, 1999). A similar phenomenon might occur during electroporation in \textit{A. baumannii}. Electroporation is also the method of choice for transposon mutagenesis where the DNA–transposase complex is directly introduced in the cell by electroporation (see below).

\textit{A. baumannii} also develops a high degree of competence for natural transformation. Unlike other naturally competent organisms, \textit{A. baumannii} develops competence soon after entry into the exponential growth phase and remains competent for a few hours after entry into the stationary phase, after which competence gradually decreases (Palmen \textit{et al.}, 1992). A recent study indicated that ~36 % of clinical isolates are naturally competent (Wilharm \textit{et al.}, 2013). Through this method, \textit{A. baumannii} could take up any plasmid DNA, linear DNA or genomic DNA from other related bacteria. Plasmid DNA can yield $\geq 10^3$ transformants $\mu$g$^{-1}$. Transformation efficacy varies depending on the isolate, and, in the case of linear DNA, on the locus of homologous recombination. The transformation efficiency varies from $10^{-3}$ to $10^{-8}$ transformants $\mu$g$^{-1}$ for linear DNA substrates. It appears that some unknown environmental signals often found in soil may boost natural transformation in certain \textit{Acinetobacter} spp. (Nielsen & van Elsas, 2001; Nielsen \textit{et al.}, 1997). Whether \textit{A. baumannii} needs additional environmental cues for the development of natural competence has not been examined critically. Nevertheless, natural transformation provides a fast and handy method to transfer DNA into the organism.

Conjugation via triparental (or four-parental) mating is an efficient method for transferring a non-conjugative but mobilizable plasmid from a donor bacterium into \textit{A. baumannii}. In this method, two \textit{E. coli} strains are used for mobilizing a plasmid into a recipient \textit{A. baumannii} (Fig. 1). The first strain acts as a helper strain that carries a self-transmissible (conjugative) plasmid (helper plasmid) encoding all the proteins necessary for transfer of itself and other mobilizable plasmids into donor or recipient cells. The donor strain carries a mobilizable plasmid (donor plasmid) that encodes the origin of transfer (oriT). The function of the helper plasmid is to provide single-strand-specific nicking activity at the oriT and target the nicked DNA to the mating channel by relaxosome function, allowing the donor plasmid to move from the donor cells to \textit{A. baumannii} recipient cells. The recognition of an oriT
sequence of a mobilizable plasmid by the helper-plasmid-encoded proteins is very specific (Hamilton et al., 2000; Lessl et al., 1992). Helper plasmids belonging to the same incompatibility group will recognize the cognate oriT of the donor plasmid. Generally, the plasmids used for triparental mating in *A. baumannii* belong to the IncP group; however, other incompatibility group plasmids can be used (Jacobs et al., 2014b). Some IncP group plasmids include derivatives of RK2, RP4 and R6. The most commonly used helper plasmid is pRK2013, which carries the transfer and mobilization activities from RK2 on a ColE1 replicon (Ditta et al., 1980, 1985; Figurski & Helinski, 1979; Singer et al., 1986). Therefore, pRK2013 itself cannot replicate in *A. baumannii*, but can mobilize a plasmid that carries oriT from RK2 (Carruthers et al., 2013; Gohl et al., 2006; Harding et al., 2013). The frequency of mobilization by triparental mating can reach as high as 10⁻¹ per recipient (Singer et al., 1986). Although not used frequently in *A. baumannii*, triparental mating can also be used as a binary cloning system. In this case, a cloning plasmid called pRK290, also a derivative of the RK2 plasmid carrying the RK2 replicon and oriT, but not self-transmissible, is used as the cloning vector (Ditta et al., 1980). Genes of interest can be cloned into this plasmid and the resultant plasmid can be transferred into *A. baumannii* with the aid of a helper plasmid such as pRK2013.

### Methods for gene inactivation and gene deletion in *A. baumannii*

The easiest and fastest method for inactivating a gene of interest is by insertional inactivation using an integration vector. Integration vectors used for this type of gene disruption must be conditional for replication to allow selection for integration into the chromosome. This is accomplished by using a plasmid that is able to replicate in a permissive host or by using a conditional replication such as a temperature-sensitive replicon. The vector must also carry a selectable marker, usually an antibiotic resistance gene. Sometimes integration vectors also contain oriT so that the construct can be transferred to other bacteria by conjugation. This is especially helpful when the other transfer methods, such as natural transformation or electroporation, are not very efficient. This is not an issue for *A. baumannii* as all the modes of gene transfer work very well. However, historically some of the vectors used for integration contain the oriT locus and are often large in size (Jacobs et al., 2014b). Integration at a particular locus on the chromosome is achieved by including a homologous sequence on the vector. If there is a homologous sequence in the plasmid, then the plasmid will integrate into the chromosome by the Campbell-type mechanism (Fig. 2). This is a single-crossover event that was originally proposed by Alan Campbell for the integration of bacteriophage into the *E. coli* genome (Campbell, 2007). For integration to work successfully, the key recombination protein RecA is required. The length of the homologous sequence is also an important factor. A fragment length of ~100 bp is needed for homologous recombination to occur and the frequency of integration is directly proportional to the length of homology. To our benefit, ColE1-derived *E. coli* plasmids do not replicate in *A. baumannii*; therefore, they can serve as integration vectors. In addition, some other *E. coli* plasmids, such as those derived from R6K, also do not replicate in *A. baumannii*. Some commonly and recently used integration plasmids for manipulation of the *A. baumannii* genome are listed in Jacobs et al. (2014b).

Integration vectors can be used for other purposes. For example, if there are two homologous sequences and the sequences are relatively close on the chromosome, a double crossover will result in integration of a resistance cassette between the chromosomal targets (Niaudet et al., 1982). This is often the straightforward method for gene deletion;
however, the major drawback with this method is the possibility of a single-crossover event. If a single crossover happens, it will simply integrate the entire plasmid without creating a true deletion.

Integration plasmids can also be used for unmarked gene deletion, i.e. deletion of a gene without inserting any resistance cassette. A schematic diagram for the overall method is shown in Fig. 3. In this method, a homologous fragment carrying either the mutated or the interrupted target gene is cloned in an integration plasmid and the integrants are selected for the single-crossover event with the help of the antibiotic resistance cassette present in the plasmid backbone. Then the integrated plasmid is allowed to excise out by a second recombination event. If the second recombination event occurs through the homology that was not used for the original integration, it will result in excision of the plasmid such that the native locus will be replaced with the mutated (or the interrupted) gene (Schweizer, 2008). The frequency of excision of the plasmid depends on the length of homology and requires the activity of the RecBCD enzyme (Zaman & Boles, 1996). For Gram-positive bacteria, it has been shown that rolling circle plasmids with a thermosensitive replicon (such as pGhost4, a pWV01 derivative; Biswas et al., 1993) are much more efficient for the second recombination event due to the production of single-stranded intermediates, which are highly recombinogenic (Biswas et al., 1993). No such conditional rolling circle plasmids have been tested for *A. baumannii*, although the pWV01 and its derivatives can replicate in *A. baumannii* (Bryksin & Matsumura, 2010; Murin et al., 2012) (unpublished). However, one can easily screen for plasmid excision by using a counter-selection marker to screen for the gene replacement event. Several counter-selection markers are available for use in *A. baumannii*. The most widely used is the sacB gene from *B. subtilis*. The sacB gene product is toxic for Gram-negative bacteria when grown in the presence of 5–10% sucrose. Therefore the transformants, which will have lost the plasmid by excision (a second recombination event) but carry the antibiotic resistance cassette, will survive on plates containing sucrose and the antibiotics. Integration plasmid pMo130-TelR (Amin et al., 2013; Hamad et al., 2009) is one such vector that contains sacB for counter-selection. This plasmid also carries a reporter gene xylE that converts pyrocatechol to 2-hydroxymuconic semialdehyde, a yellow-coloured substance (Fig. 4). Some other markers for counter-selection are pyrE that converts 5-fluoroorotic acid to 5-fluoro-UMP (Redder & Linder, 2012), gdmP (protease) that converts pregallidermin to gallidermin (Prax et al., 2013) and tdk that confers sensitivity to the base analogue azidothymidine (Metzgar et al., 2004). If the disrupted gene construct contains a selectable marker, then a direct selection for the marker is possible (Fig. 5). One can then screen the transformants for the loss of plasmid (suicide)-encoded marker to identify the double-crossover events (Fig. 5). Other vectors with improved cloning strategies such as Gateway-based
cloning or direct blunt-end cloning are also available both for cloning and gene inactivation (Eijkelkamp et al., 2011a; Oh et al., 2015).

Integration plasmids are also used for reporter gene fusion. In this case, a reporter gene is placed under the promoter of a target gene. After integration the reporter gene will be expressed from the promoter of interest in the chromosome. Several reporter genes, such as lacZ (β-galactosidase), gusA (β-glucuronidase), xylE (catechol 2,3-dioxygenase), lux (luciferase) or gfp (green fluorescent protein), can be used in A. baumannii (Davison, 2002).

Another utility for the integration plasmids is to tag a protein of interest at the C terminus. In this case the plasmid contains a homologous fragment carrying a tag (such as His6, FLAG or Myc), which is inserted in-frame at the end of the gene by removing the stop codon. After the single-crossover integration, the target gene will be tagged at the C terminus (Fig. 6). This strategy is particularly useful for tagging genes that are essential or whose expression level cannot be altered. Moreover, with the help of commercially available antibodies against the tags, it is possible to estimate the expression level of the tagged proteins in the cell. One can also purify the tagged protein from the cell by affinity chromatography (such as nickel-nitrilotriacetic acid chromatography for His-tagged protein).

**Recombineering systems for A. baumannii**

Recombineering is an in vivo system originally developed in E. coli for gene replacement purposes. Recombineering is based on homologous recombination that allows precise insertion, deletion or alteration of any sequence in the genome (Sharan et al., 2009). Linear dsDNAs (such as PCR fragments) or single-stranded oligonucleotides are introduced in the cell to provide the homologous substrates for genetic modification. Phage-encoded homologous
recombination proteins such as the lambda Red system or the RecET system from Rac prophage catalyse the process. The lambda Red system produces the phage-derived Gam, Bet and Exo proteins (Murray, 2006). The function of the Gam protein is to protect linear DNA from exonuclease degradation by the RecBCD enzyme. The Exo protein displays a progressive 5′ → 3′ exonuclease activity that creates an ssDNA overhang to which the Bet protein binds and promotes annealing of complementary DNA strands. The synergistic activity of Bet and Exo leads to insertion of linear DNA at the desired target site producing the recombinant. The RecET system is analogous to the Red system, where RecE is a 5′ → 3′ dsDNA exonuclease that uses a processive mode of digestion and therefore is very similar to lambda Exo (Shiraishi et al., 2002). However, RecT is an ssDNA annealing protein that is similar in both structure and function to the lambda Bet protein.

Although the lambda Red system works very efficiently in many Gram-negative organisms, including Agrobacterium (Hu et al., 2014), it has been reported that the E. coli lambda Red system does not work in A. baumannii and expression of the Red system might be toxic to A. baumannii (Tucker et al., 2014). However, a RecET homologue has been identified in one of the sequenced A. baumannii strains (IS-123). This system, which was named RecET, when overexpressed from a multicopy plasmid promotes recombination (Fig. 7) (Tucker et al., 2014). The recombination efficiency was highest when the homology was 125 bp and, depending on the target gene, the range varied from $10^{-9}$ to $10^{-8}$ per viable cell (Tucker et al., 2014). However if the homology is 75 bp, RecET-mediated recombineering is not productive at all in A. baumannii. Although the recombineering system works in A. baumannii, due to its low efficiency, the current system cannot be applied for unmarked gene deletion or for the introduction of mutation in the genome.

Methods for transposon mutagenesis in A. baumannii

Transposon mutagenesis is a very powerful laboratory tool that facilitates genome-scale studies of gene function. It offers a rapid and economical means of generating large numbers of independent insertions in the target genome with little experimental manipulation. Transposons that are developed for other Gram-negative bacteria such as E. coli, Pseudomonas aeruginosa and Vibrio cholerae work very well in A. baumannii (Cameron et al., 2008; Goryshin et al., 2000; Liberati et al., 2006). The two most commonly used transposon mutagenesis systems are derived from Tn5 and hinari1 (a mariner transposon). Other transposons, such as Tn3171 or Tn10, have also been used in various Acinetobacter spp. with some degree of success (Ely, 1985; Leahy et al., 1993). The EZ :: TN Transposome system, particularly EZ :: TN <R6K::ori/KAN-2> that is based on Tn5, which has a transposition frequency 1000-fold higher than the WT Tn5 (Goryshin et al., 2000), is widely used in A. baumannii and other Acinetobacter spp. (Clemmer et al., 2011; Jacobs et al., 2010; Smith et al., 2007; Tomaras et al., 2003). The EZ :: TN Transposome is a nucleoprotein complex in which the transposase is bound to the inverted repeat sequences present at the end. The main advantage of the EZ :: TN system is that the complex
is directly introduced into *A. baumannii* by electroporation and there is no need for delivery by suicide vector or conjugation. The frequency achieved through this system can be very high. In one report, as many as 2% of the colonies recovered without antibiotic selection after electroporation were mutants (Dorsey *et al.*, 2002). Several Tn5 delivery plasmids are available that can be used instead of the EZ::TN system (Fig. 8). To obtain a 95% saturation mutagenesis, one would require between 12,000 and 42,000 mutants depending on the estimation method used (Phogat *et al.*, 2001). With the high frequency of Tn5-mediated transposition, saturation mutagenesis in *A. baumannii* is easily achievable. Mapping of the insertion site is also very straightforward with the EZ::TN system. In the case of the EZ::TN < R6K<sub>ori</sub>/KAN-2 > system, a direct cloning approach termed ‘rescue’ cloning can be used. This transposon contains an *E. coli* origin of replication (R6K<sub>ori</sub>) that requires the π-protein. When genomic DNA from the mutant is appropriately restricted and self-ligated and transformed into an *E. coli* strain expressing the π-protein (encoded by *pir*), ‘rescued’ clones containing plasmids are produced. These plasmids are used for sequencing using transposon specific primers. Two other methods have been used to identify the insertion site: direct PCR using genomic DNA and random amplification of terminal ends PCR. Both are single-primer PCRs and, as they do not require cloning, they are therefore relatively fast.

A himar1-based transposon mutagenesis system, MAR2xT7, which was developed originally for *P. aeruginosa*, has also been adapted for use in *A. baumannii* (Liberati *et al.*, 2006; Peleg *et al.*, 2008b). The transposon is delivered to this organism through conjugation using a helper plasmid such as pRK2013. The frequency of transposon mutagenesis is

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**Fig. 6.** Strategies for reporter gene fusion and gene tagging using integration vectors. (a) In gene fusion, a fragment carrying a promoter region of the target gene is either transcriptionally or translationally fused with a reporter gene (hatched arrow). Upon integration, the reporter gene will be expressed from the native promoter. (b) During gene tagging, the 3′ end of the gene is translationally fused with a suitable tag (solid circle) and upon integration the tagged protein will be expressed instead of the native protein.
also very high with MAR2xT7. One of the advantages of using MAR2xT7 is to allow genetic footprinting of the mutants with transposon-site hybridization (TraSH) analysis – a method for tracking mutants in the library under various conditions. Two T7 promoters, placed at both ends of MAR2xT7 (Fig. 9), can be used for identification of the insertion site by linker-ligation-mediated PCR amplification using the genomic DNA. The PCR products obtained can be used for transcription using T7 polymerase and the RNA products can be used for making probes for hybridization to microarrays to identify the amplified genomic fragments, and thus the individual mutants present in the library. The MAR2xT7 transposon has been used in A. baumannii to identify genes necessary for interaction with Candida albicans. A derivative of the himar1 transposon was recently used in conjunction with insertion sequencing to identify A. baumannii genes required for persistence in the lung (Wang et al., 2014).

In addition to their use in genome-wide mutagenesis, transposons can be used as molecular tools for facilitating complementation, expression analysis and molecular tagging. A Tn7-based system has been developed for single-copy gene integration in many Gram-negative bacteria, including A. baumannii (Choi et al., 2005; Kumar et al., 2010). Unlike other transposons, Tn7 integrates in both a site and orientation-specific manner at neutral attachment sites (attTn7, 24 bp) generally located downstream of glmS (glucosamine 6-phosphate synthetase). Most bacterial genomes contain a single glmS gene and therefore a single insertion site for Tn7. Interestingly, although A. baumannii encodes two glmS genes, it encodes only one attTn7 and thus allows for a single integration (Kumar et al., 2010). The commonly used Tn7 vector (mini-Tn7) does not encode transposase and therefore the activity must be supplied by another helper plasmid. However, mini-Tn7 contains two flippase recognition target (FRT) sites for flippase (FIp)-mediated site-specific recombination that allows for excision of the antibiotic resistance marker (aacC1) to create the unmarked insertion. A schematic diagram for the insertion of genes through Tn7-based systems in A. baumannii is shown in Fig. 10.

Transposons are also useful for expression analysis. Several mini-Tn5-based systems have been developed for Gram-negative bacteria. These constructs encode a variety of promoterless reporter genes (phoA, lacZ, gfp and lux) to generate transcriptional or translational fusions to the inactivated genes. In addition, transposons may also contain outward-facing inducible promoters to avoid polar mutations and thus can be used for the identification of essential genes under non-inducing conditions (Judson & Mekalanos, 2000). Furthermore, like mini-Tn7, mini-Tn5
A. baumannii

As a result of its clinical significance, the vast majority of studies on A. baumannii are focused mainly on the emergence and nature of multidrug resistance. As research on the pathogenesis of A. baumannii advances and more complete genome sequences become available, the need for sophisticated and powerful genetic tools for functional genomic studies becomes necessary. Until recently, genetic tool development was primarily centred towards a few important Gram-negative pathogens such as Agrobacterium, Pseudomonas and Vibrio. Some of the tools developed for these organisms can be applied directly to the study of A. baumannii (Davison, 2002). However, because of the inherent genetic differences, the tools developed for other Gram-negative bacteria are often not adaptable for A. baumannii. Therefore, there is a need for the development of Acinetobacter-specific tools to study the clinical isolates. The choice of available antibiotic resistance markers is also a limiting factor, as it depends largely on the susceptibility of the isolate. Only a handful of antibiotic resistance markers are available to use in A. baumannii and recycling of these few valuable markers is necessary when multiple genes are targeted for modification in the same cell. Site-specific recombination is an efficient method that allows removal of antibiotic resistance markers from the genome. The two most commonly used methods are the FLP/FRT system from yeast (Schweizer, 2003) and the Cre/loxP system from bacteriophage (Marx & Lidstrom, 2002). Whilst the former system has been applied to several Acinetobacter spp., including A. baumannii, this system is not very efficient when multiple gene deletions are desired. When multiple gene deletions are required, the Cre/loxP system is ideal as the Cre recombinase can utilize two mutated loxP sites to generate an inactive loxP site that is no longer recognized by Cre. Thus, the system can be used repeatedly to delete multiple genes in a given strain (Banerjee & Biswas, 2008). Although the Cre/loxP system has not yet been adapted for A. baumannii, a thermosensitive derivative of the pWV01 plasmid carrying Cre recombinase (pCrePA; Pomerantsev et al., 2006) is available and can be used directly in A. baumannii without the need for further modification.

The MDR phenotype poses another difficulty in manipulating the MDR strains and necessitates the use of non-antibiotic resistance markers. Two such markers, tellurite and heavy metal resistance, have already been used in A. baumannii (Amin et al., 2013). Other non-antibiotic resistance markers, such as triclosan resistance, baiaphos (or phosphinothricin) resistance and glyphosate resistance, have been used in various Gram-negative bacteria such as Burkholderia and Pseudomonas, but these markers have never been used in A. baumannii (Davison, 2002). Both integrating plasmids and transposons containing these non-antibiotic markers are available, and can be adapted to A. baumannii either directly or with very little modification.

It is noteworthy to mention that the ResET-mediated recombineering system is functional in A. baumannii (Tucker et al., 2014). However, the system requires much improvement to accommodate shorter flanking fragments,
to allow recombination without any selection and to permit insertion of single amino acid changes in the genome. Though the lambda Red system does not function in A. baumannii, it is worth modifying the system so that it can be successfully adapted to A. baumannii. Alternatively, a different RecET system, perhaps from another Acinetobacter sp., would be helpful to develop a more efficient and powerful recombineering tool.

The transposon mutagenesis systems based on Tn5 or mariner transposons are highly efficient in A. baumannii. Several mutant libraries have been constructed in highly virulent clinical and laboratory isolates (Harding et al., 2013; Jacobs et al., 2014a; Umland et al., 2012; Wang et al., 2014). Some of these libraries are not comprehensive, whilst others are. In the case of a widely used A. baumannii isolate, ATCC 17987, at least two mutant libraries have been generated and one of them contains ~150 000 unique insertions, thereby surpassing 95% coverage (Smith et al., 2007; Wang et al., 2014). This particular library has been used for insertion sequencing (INSeq, a deep-sequencing method to comprehensively identify the insertion sites) to reveal novel virulence factors required for A. baumannii persistence (Goodman et al., 2009). Similar high-density libraries in other MDR clinical isolates would be necessary to systematically understand the pathogenesis of this organism.

Acknowledgements

This work was made possible by a Fulbright–Nehru Senior Research Fellowship award to I. B. The author would like to thank Dr V. Balaji (Christian Medical College, Vellore, India) for helpful discussion.

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