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An optimized method for suicide vector-based allelic exchange in *Klebsiella pneumoniae*

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ABSTRACT

Klebsiella pneumoniae is an important and versatile bacterium that can be found in diverse environments and is also a frequent cause of human infections. Limited data exists on the mechanisms of interaction between *K. pneumoniae* and the human host and of adaptations to other environments. Coupled with the high genetic diversity of this species, these factors highlight the necessity for substantial further *K. pneumoniae*-focused molecular genetics studies.

In this report we describe a simple and efficient experimental protocol for suicide vector-based allelic exchange in *K. pneumoniae*. The protocol has been validated by mutating multiple loci in four distinct *K. pneumoniae* strains, including highly capsulated and/or multi-antibiotic resistant clinical isolates. Three key enhancements are reported:(1) Use of pDS132-derived conjugative plasmids carrying improved cloning sites, (2) Performance of *sacB* counterselection at 25 °C as opposed to higher temperatures, and (3) Exploitation of Flp-recombinase-mediated deletion of FRT (Flp recombinase target) flanked resistance cassettes to allow for reiterative manipulations with a single selectable marker. This study also highlights a problem that may be encountered when the *aacC1* gentamicin resistance marker is used in *K. pneumoniae* and suggests alternative markers.

The protocol developed in this study will help investigate the plethora of uncharacterized genes present in the *K. pneumoniae* pan-genome and shed further light upon clinically and industrially important phenotypes observed in this ubiquitous species.

1. Introduction

Klebsiella pneumoniae are clinically and economically important members of the *Enterobacteriaceae* family. This species accounts for a significant proportion of community- and hospital-acquired urinary, respiratory, wound and bloodstream infections (Podschun and Ullmann, 1998). Over the last decade *K. pneumoniae* infections have become more difficult to treat due to increased prevalence and geographical spread of multi-antibiotic resistant strains (Rice, 2010). Additionally, highly invasive and pathogenic pyogenic liver abscess-causing strains of *K. pneumoniae* have emerged and are rapidly spreading from an initial focus in East Asia (Tsai, 2008). On the other hand, not all *K. pneumoniae* strains are pathogenic and some have been used to improve crop yields by nitrogen fixation or to produce 1,3-propanediol and other chemicals (Zhang et al., 2007; Fouts et al., 2008).

A better understanding of *K. pneumoniae* pathogenicity and industrially important phenotypes is essential for improved clinical management and industrial applications, respectively. A common strategy to identify gene or operon function is to delete or disrupt the region of interest using allelic exchange and to then carry out phenotypic analysis on the derived mutant, a technique referred to as reverse genetics. Excellent descriptions of this technique and matching genetic tools applicable for a range of bacteria have been published (Reyrat et al., 1998; Blomfield et al., 1991).

Many genetic tools have been developed for allelic exchange in *Escherichia coli* and other Gram negative bacteria, and several of these systems have been adapted for use in *K. pneumoniae*. Lambda Red recombination, a method whereby bacteria over-expressing the phage lambda Red recombination system are transformed with linear DNA fragments flanked by short homologous sequences which then recombine into the host chromosome (Datsenko and Wanner, 2000), has been used successfully in *K. pneumoniae* (Clements et al., 2007; Struve et al., 2008). However, its applicability is limited to selected strains as there is considerable strain-to-strain variation in both capsule thickness and transformation efficiency (Fournet-Fayard et al., 1995). We have also found that some strains are unable to support the commonly used lambda Red recombination plasmid pKD46 (J. J. van Aartsen and K. Rajakumar, unpublished results). Similarly, low transformation efficiency limits the effectiveness of suicide plasmids such as pKOV which requires electrotransformation (Rosen et al., 2008).

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To circumvent transformation related issues some investigators have used conjugation-based methods for delivery of suicide vectors. pUT-based plasmids can be conjugated into *K. pneumoniae* strains and readily suicide in this species, allowing for insertional disruption of chosen genes by single-homologous recombination (Chuang et al., 2006). However, following single-crossover two truncated copies of the target gene remain, potentially masking effects that would be associated with complete loss or disruption of the target gene. Moreover, larger deletions spanning entire operons, or even genomic islands, cannot be generated by this approach, and targeted disruption of genes within operons imposes likely downstream polar effects.

Here we report the construction of two derivatives of the lambda *pir*-dependent conjugative suicide vector pDS132 (Philippe et al., 2004) that offer added cloning utility and an accompanying conjugation and screening protocol for efficient allelic exchange in *K. pneumoniae* that can be used to knockout single genes and/or entire operons. We also describe a rapid method for the construction of mutant allele cassettes and a specific problem encountered with the commonly used *aacC1* gentamicin resistance cassette. Finally, we discuss the application of Flp recombinase-mediated deletion of FRT (Flp recombinase target) flanked antibiotic resistance cassettes in *K. pneumoniae* for the construction of markerless mutants.

2. Methods

2.1. Bacterial strains and growth conditions

The strains and plasmids used in this work are described in Table 1. Clinical *K. pneumoniae* isolates were obtained from the Leicester Royal Infirmary and were stored at −20/−80 °C in brain heart infusion broth with 30% glycerol. Bacterial strains were routinely grown at 37 °C in LB broth or agar supplemented with appropriate antibiotics, unless otherwise specified. When required, *E. coli* were grown on media supplemented with 100 μg ml^{-1} ampicillin, 50 μg ml^{-1} kanamycin, 30 μg ml^{-1} chloramphenicol and/or 13 μg ml^{-1}gentamicin. When required, *K. pneumoniae* were grown on media supplemented with ampicillin (250 μg ml^{-1}) and/or gentamicin (9 μg ml^{-1}), unless otherwise specified.

2.2. Construction of pDS132-based derivatives

A 5.2 kb amplicon produced by inverse PCR with KOD HS DNA Polymerase (Merck Chemicals), the primers pDS132_cw_NotI and pDS132_acw_NotI (Table 2) and pDS132 as template was cloned into pJET1.2 (Fermentas), according to manufacturer's instructions, to produce pJTOOL-1CJ. pJTOOL-1CJ was then digested with NotI and re-ligated to remove the pJET1.2 backbone and produce pJTOOL-1 (Fig. 1A). The pJTOOL-1 sequence has been deposited in Genbank (JF756692).

pJTOOL-3 was constructed from both pDS132 and pBluescript II KS+ (Fig. 1A). The 5.2 kb pDS132-derived fragment was amplified from pDS132 using KOD HS DNA polymerase and primers pDS132_acw_SceI and pDS132_cw. Similarly, a 0.2 kb pBluescript II KS+ fragment was produced using primers T7_SceI and T3_pDS132_cw. Both fragments were then recombined using a modified SLIC (Sequence and Ligation–Independent Cloning) method (Li and Elledge, 2007). Briefly, both fragments were subjected to 3′–5′ T4 DNA polymerase (New England Biolabs) exonuclease digestion at room temperature for 30 min, after which the reaction was stopped by adding dCTP. The two fragments were subsequently annealed by mixing a 1:1 molar ratio and incubating for 30 min at 37 °C in 1×STE buffer (10 mM Tris–HCl, pH 8.0, 0.1 M NaCl, 1 mM EDTA) supplemented with 20 ng of RecA (Epicentre). The ligation was then transformed into *E. coli* DH5α harboring pRDH137 and recombinants were selected on plates supplemented with kanamycin and chloramphenicol. Plasmid preparations (OmegaBiotek EZNA Plasmid Mini Kit I, VWR) obtained from these clones contained a mixture of pJTOOL-3 and pRDH137 that was subsequently separated by transforming the mix into *E. coli* CC118λ*pir* and plating on LB plates supplemented with chloramphenicol to select for pJTOOL-3 alone. The pJTOOL-3 sequence has been deposited in Genbank (JF756693).

2.3. Construction of suicide vectors

Mutant alleles were constructed to delete the *K. pneumoniae fim* operon (*fim*::*aacC1*$_{FRT}$) and *fimK* gene (*fimK*::*aacC1*$_{FRT}$). Mutant allele cassettes were generated by spliced overlap extension-PCR (SOE-PCR) and consisted of a left homologous flank, an antibiotic cassette and a right homologous flank (Choi and Schweizer, 2005). The selected flanks were 800 to 1000 bp long and were homologous to sequences upstream and downstream of the region targeted for deletion. All SOE-PCR fragments were generated using KOD HS DNA Polymerase, according to manufacturer's instructions.

The *fim*::*aacC1*$_{FRT}$ cassette was constructed as follows. Initially, the FRT-flanked gentamicin resistance cassette (*aacC1*$_{FRT}$) from pUC18R6K-mini-Tn7T-Gm was amplified using GmF and GmR. The left and right flanking sequences were amplified from *K. pneumoniae* KR116 using primer pairs opfim_lf_NotI/opfim_lr_GmF (932 bp) and opfim_rf_GmR/opfim_rr_NotI (918 bp), respectively. Primers opfim_lr_GmF and opfim_rf_GmR contained 20 bp overlaps at their 5′ ends with primers GmF and GmR, respectively. Secondly, 10 ng of the *aacC1*$_{FRT}$ fragment was mixed with 10 ng of the left flank and PCR amplified using primers opfim_lf_NotI and GmR, producing a left flank-*aacC1*$_{FRT}$ fragment. In a similar manner, a right flank-*aacC1*$_{FRT}$ fragment was produced using primers opfim_rr_NotI and GmF. Finally, 10 ng of the left flank-*aacC1*$_{FRT}$ and right flank-*aacC1*$_{FRT}$ PCR products were mixed and subjected to amplification using primer pairs opfim_lf_NotI and opfim_rr_NotI, producing *fim*::*aacC1*$_{FRT}$. After digestion with NotI the 2.8 kb SOE-PCR product was cloned into the NotI restriction site of pJTOOL-1 to produce pJKO-5b.

The *fimK*::*aacC1*$_{FRT}$ cassette was constructed in a similar manner, with the primers fimK_lf_NotI/fimK_lr_GmF and fimK_rf_GmR/fimK_rr_NotI

Table 1
Strains and plasmids used in this study.

Strains/plasmids[a]	Relevant properties[b]	Reference
Strains		
Ec DH5α	Laboratory cloning strain	Promega
Ec CC118λ*pir*	Cloning strain able to host lambda *pir*-dependent plasmids	Simon et al. (1983)
Ec S17-1λ*pir*	Conjugative strain able to host lambda *pir*-dependent plasmids	Simon et al. (1983)
Kp KR116	Clinical *Kp* bloodstream infection isolate	This work
Kp KR161	Clinical *Kp* bloodstream infection isolate	This work
Kp KR162	Clinical *Kp* bloodstream infection isolate	This work
Kp KR173	Clinical *Kp* bloodstream infection isolate	This work
Plasmids		
pBluescript II KS+	Amp; high copy number cloning vector	Stratagene
pDS132	Cml, lambda *pir*-based suicide vector	Philippe et al. (2004)
pJTOOL-1	Cml; pDS132 derivative with NotI restriction site	This work
pJTOOL-3	Cml; pDS132 derivative with pBluescript II KS + multiple cloning site	This work
pRDH137	Kan; pTH18ks1 harboring the *pir* gene	Haigh R., unpublished
pUC18R6K-mini-Tn7T-Gm	Amp, Gen; possesses an *aacC1*$_{FRT}$ resistance cassette	K. Choi et al. (2005)
pFLP2	Amp; encodes FLP recombinase	Hoang et al. (1998)
pJKO-1b	Cml, Gen; pJTOOL-1 with Δ*fimK*::*aacC1*$_{FRT}$ within NotI site	This work
pJKO-5b	Cml, Gen; pJTOOL-1 with Δ*fim*::*aacC1*$_{FRT}$ within NotI site	This work

[a] Strain abbreviations are as follows: *Ec*, *Escherichia coli*; and *Kp*, *Klebsiella pneumoniae*.
[b] Antibiotic resistance phenotypes are abbreviated as follows: Amp, ampicillin; Cml, chloramphenicol;Kan, kanamycin; and Gen, gentamicin.