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Knock-out or knock-in? Converting a SacB-based gene disruption system for site-specific chromosomal integration in *Pseudomonas syringae* pv. *tomato* DC3000

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ABSTRACT

Recent advances in next generation sequencing technology allow us to retrieve the whole genome sequence of a requested bacterium in less than a day. Thus, development of quick, easy and efficient means to systemically analyze the functions of all genes is required in the post-genome era. Here, a procedure of finding a suitable chromosome integration site and developing a gene disruption system into a knock-in system in Gram-negative bacteria is proposed. As a proof of concept, we successfully modified a *sac*B-based gene knock-out strategy into a site-specific gene integration system to deliver a DNA fragment into the genome site between 313,520 bp and 313,521 bp of the model phytopathogenic bacterium, *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000. The expression levels of *avrPtoB* and *hcp2* integrated using this method exhibited steady and similar expression levels as those in the wild type. In the future, this concept could allow us to easily develop gene replacement and delivery systems at the same time using a counter-selectable suicide vector-based allelic exchange strategy, and facilitate functional genomics studies of any bacterium whose genome has been sequenced.

1. Introduction

Abundant information generated from bacterial genome sequencing projects enables researchers to propose possible mechanisms for diverse behaviors of each microorganism. In the post-genome era, reverse genetics becomes a straightforward approach for characterization of gene functions in detail, which usually uses a standard procedure of either random mutagenesis followed by complementation of the mutant (loss-of-function) or heterologous expression of the target gene in alternate organisms (gain-of-function) to assess certain phenotypes (Gilchrist and Haughn 2010; Lawson and Wolfe 2011). Both strategies involve effective gene expression systems, which can be categorized into plasmid-mediated and chromosomal integration-based ones, depending on the location of gene-of-interest in the target cells (Yu et al. 2000; Ghosh and Van Duyne 2002; Hayes 2003; Choi and Kim 2009). The utilization of engineered plasmids to express target genes has become indispensable for studying gene functions in diverse bacteria, but disadvantages related to variation in plasmid copy number, plasmid instability, and limited types of selectable marker may restrict its application. Also, continuous antibiotic pressure is usually required to maintain the recombinant plasmids. If the phenotype assessment has to be accomplished inside the host tissues or in complex environments (such as rhizosphere), where antibiotic selection is impossible, the gene activities might be underestimated due to plasmid loss (DebRoy et al. 2012; Imanaka and Aiba 1981).

Fortunately, these problems can be solved with the advent of approaches for chromosomal integration, which can be achieved by homologous recombination, site-specific recombination, or transposon-mediated gene integration (Ghosh and Van Duyne 2002; Hayes 2003; Choi and Kim 2009). Among these strategies, transposon-mediated gene integration does not require homologous sequences, so it is extensively used to create random mutants. However, transposition in a random fashion actually hampers its application as a delivery system for gene integration except those which can be integrated at a defined site, such as mini-Tn7 (Bao et al. 1991; Choi and Kim 2009).

Pseudomonas syringae pv. *tomato* (*Pst*), the causing agent of tomato speck disease, has become one of the most studied plant pathogens due to its economic importance and distinct interactions with its hosts (Buell et al. 2003). The genome sequence of *Pst* strain DC3000 published in 2003 has facilitated the genome-wide studies to identify novel genes involved in pathogenesis or virulence (Fouts et al. 2002; Petnicki-Ocwieja et al. 2002). To accelerate functional studies, an effective *sacB*-

Abbreviations: Pst, *Pseudomonas syringe* pv. *tomato*; LP, landing pad; KBM, King's B medium; HMM, *hrp*-inducing minimal medium

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based counter selection and a *FRT*/FLP system has been successfully applied to *Pst* DC3000 for making markerless deletions of the type III effectors (Wei et al. 2007; Kvitko and Collmer 2011). In addition, RecTE-dependent recombineering of *P. syringae* was also reported which has potential as a powerful alternative means for site-directed mutagenesis of particular loci in *Pst* DC3000 (Swingle et al. 2010). However, utilization of broad-host-range plasmids (Keen et al. 1988; Labes et al. 1990; Bauer and Collmer 1997) is still a prevalent method to complement deleted genes or heterologously express genes of interest in *Pst* DC3000 although problems associated with this approach are increasing. For example, unable to maintain the recombinant plasmids (or cosmids) in the transformed cells in the absence of antibiotic selection has been reported in some studies of type III secretion effectors in *Pst* DC3000 in the pathogenicity assays, which may cause underestimation of the gene function during bacterial growth *in planta* (Fouts et al. 2003; Lin and Martin 2005).

Two-step allelic exchange has been successfully used for making precise, unscarred and unmarked mutations in *Pseudomonas* spp. (Haapalainen et al. 2012; Hmelo et al. 2015). Although a protocol for the introduction of an exogenous genetic element into the chromosome in *P. aeruginosa* using similar techniques has been developed, construction of a plasmid for such allelic exchange is elaborate, and involves a conventional cloning procedure with three primer sets and an overlapping PCR (Hmelo et al. 2015). Here, a genomic region from 312,821 bp to 314,157 bp of *Pst* DC3000, which is suitable for integration of any DNA fragment, was cloned into the plasmid pK18mobsac, which was then converted into a general vector for site-specific chromosomal integration of *Pst* DC3000. This new vector allows us to revisit the virulence and avirulence activities of AvrPtoB, and the interbacterial competition ability of the type VI hallmark protein Hcp2 (Haapalainen et al. 2012), and obtain more satisfactory data.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Pst* DC3000 strains were grown in King's B medium (KBM) (King et al. 1954) supplemented with appropriate antibiotics at 28 °C unless otherwise stated. *Escherichia coli* strains were grown in Luria-Bertani medium with appropriate antibiotics at 37 °C. The concentrations of antibiotics used in the selective media were as follows: kanamycin, 50 μg/mL; gentamycin, 10 μg/mL; rifampicin, 50 μg/mL; and tetracycline, 10 μg/mL. For detection of different effectors, *Pst* DC3000 was grown in *hrp*-inducing minimal medium (HMM) as described previously (Huynh et al. 1989; Lin et al. 2006).

2.2. Construction of pK18msLP

pK18mobsac, which has been used for *sacB*-based gene disruption in many Gram-negative bacteria (Schafer et al. 1994), was modified to generate pK18msLP. First, DNA fragments containing genomic region 312,821 bp to 313,520 bp and 313,521 bp to 314,157 bp were PCR amplified using primer pairs LP_1-1/LP1-2 and LP_1-3/LP1-4 listed in Table S1 in the Supplementary materials. After treating with appropriate restriction endonucleases, these two fragments were subsequently cloned into the *Eco*RI and *Hind*III sites of pK18mobsac, respectively. Next, for efficient transcriptional termination of the gene inserted at the site between 313,520 bp and 313,521 bp, DNA fragment containing the *rrn*B T1 terminator sequence was PCR amplified using primers *Pst*I_rrnB T1-F/PstI_rrnB T1-R and pCPP5242 as a template, and cloned into the *Pst*I site of the vector obtained from the previous step to generate pKms18LP (Fig. 1C). To integrate *avrPtoB* and *hcp2* into the *Pst* DC3000 genome, a DNA fragment containing each gene and its native promoter was PCR amplified from *Pst* DC3000 genomic DNA using appropriate primer pairs (Table S1). Each fragment was then cloned into the corresponding site of pKms18LP to generate

Table 1
Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	Source
Pst DC3000 strains		
DC3000	Wild type; Rif^r	Cuppels, 1986
Δ*avrPto*	Δ*avrPto*::ΩSp^r/Sm^r; Rif^r, Sp/Sm^r	Ronald et al. 1992
Δ*avrPto*Δ*avrPtoB*	Δ*avrPto*::ΩSp^r/Sm^r; in-frame deletion of *avrPtoB*; Rif^r, Sp/Sm^r	This study
Δ*hcp1*	in-frame deletion of PSPTO_2539 (*hcp1*); Rif^r	Haapalainen, 2012
Δ*hcp2*	in-frame deletion of PSPTO_5435(*hcp2*); Rif^r	Haapalainen, 2012
Δ*hcp1/2*	in-frame deletion of PSPTO_2539 and PSPTO_5435; Rif^r	Haapalainen, 2012
Pst DC3000 complementary strains		
Δ*avrPtoB* + ev	Δ*avrPtoB* carrying pCPP45 (empty vector); Rif^r, Tet^r	This study
Δ*avrPtoB*Cp	Δ*avrPtoB* carrying p*avrPtoB*; Rif^r, Tet^r	This study
Δ*avrPtoB*Cg	Δ*avrPtoB* with *avrPtoB* under *hrp* promoter integrated into genome; Rif^r	Lin and Martin 2005
Δ*hcp2* + ev	Δ*hcp2* (Pto_5435) carrying pCPP45; Rif^r, Tet^r	This study
Δ*hcp1/hcp2* + ev	Δ*hcp1/2* (Pto_2539/5435) carrying pCPP45; Rif^r, Tet^r	This study
Δ*hcp2*Cp	Δ*hcp2* (Pto_5435) carrying p*hcp2*; Rif^r, Tet^r	This study
Δ*hcp1/2*Cp	Δ*hcp1/2* (Pto_2539/5435) carrying p*hcp2*; Rif^r, Tet^r	This study
Δ*hcp2*Cg	Δ*hcp2* with *hcp2* under native promoter integrated into genome; Rif^r	Haapalainen, 2012
Δ*hcp1/2*Cg	Δ*hcp1/2* with *hcp2* under native promoter integrated into genome; Rif^r	Haapalainen, 2012
E. coli strains		
S17–1 λ*pir*	*recA pro hsdR RP4–2-Tc::Mu-Km::Tn7*λ*pir,* Tp^r	Laboratory stock
MG1655	K-12 strain; F$^-$ lambda$^-$ *ilvG*$^-$ *rfb*-50 *rph*-1	Dr. Whei-Fen Wu, National Taiwan Univ.
BL21(DE3)	F$^-$ *dcm ompT hsdS* (r_B^- m_B^-) *gal* λ(DE3)	New England Biolabs
Plasmids		
pΔPTO3087	pK18mobsac derivative for deletion of *avrPtoB* (PTO3087) in *Pst*DC3000; Km^r	This study
pCPP45	Broad-host-range vector with RP4 *par* region; Tet^r	D. W. Bauer, Cornell University
p*avrPtoB*	pCPP45 expressing *avrPtoB* under its native promoter; Tet^r	Lin and Martin 2005
p*hcp2*	pCPP45 expressing *hcp2* under its native promoter; Tet^r	This study
pK18mobsac	RP4 *mob*; suicide vector with *sacB* gene; Km^r	Schafer et al. 1994
pK18msLP	For landing pad idea; Km^r	This study