Journal of Microbiological Methods 144 (2018) 47–52

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

A temperature-sensitive replicon enables efficient gene inactivation in *Pseudomonas aeruginosa*

Ramadevi Prathapam, Tsuyoshi Uehara*

Infectious Diseases Area, Novartis Institutes for BioMedical Research, 5300 Chiron Way, Emeryville, CA 94608, United States

ARTICLE INFO

Keywords:
Gene inactivation
Pseudomonas aeruginosa
Temperature-sensitive replicon

ABSTRACT

Tools to enable genome editing are essential for understanding physiology. Here we report a gene replacement method in *Pseudomonas aeruginosa* using a temperature-sensitive replicon plasmid that does not require mating or isolation of a merodiploid intermediate. This approach was validated by replacing the non-essential *ampD* gene with a gentamicin resistance cassette. In addition *lpxA* and *lpxD*, both located in a complex gene cluster including multiple downstream essential genes, were inactivated when complemented by each target gene *in trans*. These strains did not grow when expression of the gene *in trans* was repressed, confirming that both genes are essential for viability. This method facilitates efficient gene inactivation in *P. aeruginosa*.

1. Introduction

Pseudomonas aeruginosa is an opportunistic Gram-negative pathogen often associated with hospitals and healthcare settings. An increasing rate of infections by multi-drug resistant *P. aeruginosa* is a serious threat (Oliver et al., 2015). In order to develop new therapies against this pathogen and elucidate mechanisms of drug action, the ability for rapid genetic manipulation is important, and multiple genetic tools have been developed in *P. aeruginosa* (Schweizer and de Lorenzo, 2004; Suh et al., 2004). Most routinely-used methods to replace chromosomal regions are based on pEX18 vector and require several steps, including mating with *E. coli*, merodiploid strain isolation and then counter selection (Hmelo et al., 2015; Hoang et al., 1998; Schweizer, 1992; Schweizer and Hoang, 1995; West et al., 1994). Although CRISPR genome engineering is developed in multiple bacteria, the application in *P. aeruginosa* has not been reported (Krishnamurthy et al., 2016). Other Pseudomonas recombineering tools such as the RED system of *E. coli* lambda phage and the cognate RecET system are described (Lesic and Rahme, 2008; Liang and Liu, 2010; Swingle, 2014), but have not been reported extensively for genome recombineering of *P. aeruginosa* (Martinez-Garcia and de Lorenzo, 2017).

Here we report the application of a temperature-sensitive (Ts) replicon vector for gene replacement in *P. aeruginosa* PA01. Ts replicon vectors have been used as powerful tools for a wide variety of genetic manipulations in *E. coli*, such as knockout mutant construction, integration of genes or reporter fusions onto the chromosome, and conditional gene expression (Emmerson et al., 2006). Based on the *P. aeruginosa* temperature-sensitive replicon plasmids reported previously (Chen et al., 2010; Silo-Suh et al., 2009), we constructed a temperature-sensitive replicon vector using the Ts mutations in the Ori1600 replication control (Rep) protein. As a test for this approach, we replaced the non-essential *ampD* gene with a gentamicin resistance cassette (Gm^r). Using this new method we were also able to make gene replacements of the *lpxA* and *lpxD* genes that reside in a complex gene cluster that has multiple essential genes downstream (in the presence of the corresponding gene *in trans*) and confirmed the essentiality of LpxA and LpxD for growth. In contrast, the pEX18-based system did not allow us to make the *lpxD*-controlled expression strain probably due to polar effects on expression of the downstream essential genes. Since this gene replacement procedure is simple and efficient, it is a useful tool for *P. aeruginosa* mutant strain construction.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Lysogeny (L) broth (Miller, 10 g/L Bacto trypton, 5 g/L Bacto yeast extract, and 10 g/L NaCl) and L agar were used for routine growth of *E. coli* and *P. aeruginosa*. Cation-adjusted MHBII broth (MHB) was

Abbreviations: Ts, temperature-sensitive; L broth, lysogeny broth; MHB, cation-adjusted Mueller-Hinton Broth II; Gm^r, gentamicin resistant cassette; Flp, *S. cerevisiae* recombinase; *frt*, Flp recombinase target; *bla*, TEM-1 β-lactamase gene; tet^r, tetracycline resistant gene; *ori*, origin of replication; ori_{1600}, *bhr* replicon; *oriT*, origin of transfer; *sacB*, *B. subtilis* levansucrase-encoding gene

* Corresponding author.
E-mail address: tsuyoshi.uehara@novartis.com (T. Uehara).

https://doi.org/10.1016/j.mimet.2017.11.001
Received 16 October 2017; Received in revised form 2 November 2017; Accepted 2 November 2017

Table 1
Bacterial strains and plasmids used or constructed in this study.

Strain or plasmid	Genotype or relevant characteristics	Reference or source
Strains		
PA01	*P. aeruginosa* prototroph strain K767	Poole (2004)
TUP0040	PA01 *ampD*::*frt*	This study
TUP0041	PA01 *lpxA*::Gm^r/pTU494 (Ptop-10::Pa*lpxA*)	This study
TUP0007	PA01 *lpxD*::Gm^r/pTU481 (Ptop-10::Pa*lpxD*)	This study
Plasmids		
pEX18Tc	Suicide vector, *pBR*, *sacB*, Tc^r	Hoang et al. (1998)
pEX18Tc-Gm	Suicide vector, *sacB*, *Tc^r*, *Gm^r*(*aacC1*)	This study
pFLP3	*pBR*, *ori1600*, *sacB*, Flp recombinase, *Tc^r*, *Ap^r*	Choi and Schweizer (2005)
pMMB206	Broad host low copy, *Cm^r*, *lacI^q*, P_{lac}::*lacZα*	Morales et al. (1991)
pUCGM	*pUC*, *Ap^r*, *Gm^r*(*aacC1*)	Schweizer (1993)
pUC18T-mini-Tn7T-Gm	*pUC*, Ap^r, *frt-Gm^r*(*aacC1*)-*frt*, *Tn7R/L*	Choi et al. (2005)
pBTK29	pMMB67EH derivative, broad host low copy, Ap^r, *lacI^q*, promoter P_{Top-10}	Stephen Lory
pTU451	pFLP3, *ori1600*-Ts (Rep[G100C, S204R])	This study
pTU487	pTU451 *ampD^{up}-frt-Gm^r-frt-ampD^{down}*	This study
pTU476	pTU451 *lpxA^{up}-Gm^r-lpxA^{down}*	This study
pTU456	pEX18Tc *lpxD^{up}-Gm^r-lpxD^{down}*	This study
pTU462	pTU451 *lpxD^{up}-Gm^r-lpxD^{down}*	This study
pTU414	pMMB206, *Cm^r* promoterP_{Top-10}::*lacZα*	This study
pTU494	pTU414 P_{top-10}::*PalpxA*	This study
pTU481	pTU414 P_{top-10}::*PalpxD*	This study

used for growth experiments and cell preparation for β-lactamase assay. Cells were grown at 37°C unless otherwise described. For selection, gentamicin was used at 10 μg/mL (*E. coli*) or 100 μg/mL (*P. aeruginosa*), and carbenicillin was used at 30 μg/mL (*E. coli*) or 100 μg/mL (*P. aeruginosa*) unless otherwise specified. L agar (no salt) was supplemented with 8% sucrose for *sacB*-mediated counter selection.

2.2. Plasmid construction

PCR was performed using Phusion High-Fidelity DNA Polymerase (New England BioLabs). *P. aeruginosa* PA01 genomic DNA or suspension of colonies was used as the template. Oligonucleotide primers used are listed in Table 2. Plasmid DNA and PCR fragments were purified using the Qiaprep spin miniprep kit (Qiagen) or the Qiaquick PCR purification kit (Qiagen), respectively. Restriction enzymes (Thermo Fisher Scientific) were used to digest plasmids or DNA fragments. Cloning was done using either Quick ligase (New England Biolabs), DNA Ligation Kit (Takara Bio USA) or GeneArt Seamless Cloning and Assembly Kit (GA) (Thermo Fisher Scientific). Top10 and DH5α competent cells (Thermo Fisher Scientific) were used for transformation. The inserted sequences of the plasmids constructed below were confirmed with sequencing using appropriate primers. DNA sequencing was performed by Elim Biopharmaceuticals (Hayward, CA) and Quintara Biosciences (Berkeley, CA). DNA sequences were aligned and analyzed with the Vector NTI software (Thermo Fisher Scientific) and the Sequencher DNA sequence analysis software (Gene Codes Corporation, Ann Arbor, MI).

pTU451 (pFLP3 Rep[Gly100Cys, Ser204Arg]^{ts}). The point mutations, G894 to T and C1208 to A, at the nucleotide positions of the pFLP3 plasmid sequence (Choi and Schweizer, 2005) [GenBank: AY597273.1] were created by QuikChange Multi Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA) using 5′-phosphorylated primers TU204 and TU205 according to the manufacturer's instruction. The plasmid that was confirmed to carry the point mutations by sequencing with primers TU136 and TU137 was named pTU451.

pEX18Tc-Gm (Suicide vector, pBR, *sacB*, Tc^r, Gm^r). The cassette including Gm^r (*aacC1*) was PCR-amplified from pUCGM (Schweizer, 1993) [GenBank ID: U04610.1] using primers E156 and E157 primers. The PCR fragment was then digested with *Bam*HI and *Pst*I, and inserted into pEX18Tc (Hoang et al., 1998) [GenBank ID: AF047519.1], generating pEX18Tc-Gm.

pTU487 (pTU451 *ampD*^{up}-frt-Gm^r-frt-*ampD*^{down}). The Gm^r cassette was PCR-amplified from pUC18T-mini-Tn7T-Gm plasmid (Choi et al., 2005) [GenBank ID: AY599232] using primers TU311 and TU312. The *ampD*-flanking DNA fragments were PCR-amplified from the PA01 genomic DNA using TU313 and TU314 primers for the upstream region and TU315 and TU316 primers for the downstream region. The three PCR products were stitched using PCR with TU313 and TU316 primers and the product was cloned into the *Eco*RI-digested pTU451 using GA, generating pTU487.

pTU476 (pTU451 *lpxA*^{up}-Gm^r-*lpxA*^{down}). The Gm^r cassette was PCR-amplified from pEX18Tc-Gm plasmid using primers TU303 and TU304. The *lpxA*-flanking DNA fragments were PCR-amplified from the PA01 genomic DNA using TU301 and TU302 primers for the upstream region and TU305 and TU306 primers for the downstream region. The three PCR products were stitched using PCR with TU301 and TU306 primers and the product was cloned into the *Eco*RI-digested pTU451 using GA, generating pTU476.

pTU456 (pEX18Tc *lpxD*^{up}-Gm^r-*lpxD*^{down}). The *lpxD* upstream DNA fragment was PCR-amplified from the PA01 genomic DNA using TU236 and TU237 primers, digested with *Hind*III and *Nsi*I, and cloned into the *Hind*III-*Pst*I site of pEX18Tc-Gm using GA. Then the constructed plasmid and the lpxD downstream fragment that was PCR-amplified from the PA01 genomic DNA using TU238 and TU239 primers were digested with *Bam*HI and *Kpn*I and GA-cloned, generating pTU456.

pTU462 (pTU451 *lpxD*^{up}-Gm^r-*lpxD*^{down}). The *lpxD*^{up}-Gm^R-*lpxD*^{down} DNA fragment was PCR-amplified from pTU456 using TU252 and TU253 primers, and GA-cloned into the *Eco*RI digested pTU451 generating pTU462.

pTU414 (pMMB206 P_{top-10}::*lacZα*). The derivative of the *tac* promoter, P_{top-10}, was PCR-amplified using primers TU12 and TU34 from pBTK29 (gift from Stephen Lory, Harvard Medical School), derivative of pMMB67EH (Furste et al., 1986). The amplified DNA fragment was digested with *Hpa*I and *Hind*III, and ligated into the *Hpa*I-*Hind*III site of pMMB206 (Morales et al., 1991), generating pTU414. The P_{top-10} promoter sequence 5′-TGTTtACATTGTGAGCGGATAACAAcATTAT contains the underlined *lacO* sequence sandwiched with the modified −10 and −35 sequences (changes shown in lower cases) of the P_{tac} promoter.

pTU494 (pTU414 P_{top-10}::*lpxA*). The *lpxA* gene was PCR amplified from PA01 genomic DNA using TU372 and TU373, digested with *Eco*RI and *Hind*III, and GA-cloned into the *Eco*RI-*Hind*III site of pTU414, generating pTU494.

pTU481 (pTU414 P_{top-10}::*lpxD*). The *lpxD* gene was PCR amplified from the PA01 genomic DNA using TU329 and TU331, digested with