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Tools for high efficiency genetic manipulation of the human pathogen *Penicillium marneffei*

Hayley E. Bugeja [1], Kylie J. Boyce [1], Harshini Weerasinghe, Sally Beard, Anne Jeziorowski, Shivani Pasricha, Michael Payne, Lena Schreider, Alex Andrianopoulos *

Department of Genetics, The University of Melbourne, Parkville, Victoria 3010, Australia

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ABSTRACT

Penicillium marneffei is an opportunistic pathogen of humans and displays a temperature dependent dimorphic transition. Like many fungi, exogenous DNA introduced by DNA mediated transformation is integrated randomly into the genome resulting in inefficient gene deletion and position-specific effects. To enhance successful gene targeting, the consequences of perturbing components of the non-homologous end joining recombination pathway have been examined. The deletion of the *KU70* and *LIG4* orthologs, *pkuA* and *ligD*, respectively, dramatically enhanced the observed homologous recombination frequency leading to efficient gene deletion. While Δ*pkuA* was associated with reduced genetic stability over-time, Δ*ligD* represents a suitable recipient strain for downstream applications and combined with a modified Gateway™ system for the rapid generation of gene deletion constructs, this represents an efficient pipeline for characterizing gene function in *P. marneffei*.

1. Introduction

Many species of fungi make excellent experimental models for exploring mechanisms governing growth, morphogenesis and development due to their ease of manipulation, at both the organismal and genetic level. Recent advances in sequencing technologies are providing a wealth of functional genomics information, necessitating high-throughput methods for characterizing the function of candidate genes identified in these studies. The ability to understand gene function requires genetic modification of the organism via DNA-mediated transformation, during which time the fate of the incoming DNA is determined by endogenous DNA repair pathways (Kwon-Chung et al., 1998).

Two main recombination pathways that repair double stranded breaks have been identified in eukaryotes; non-homologous end joining (NHEJ), which ligates DNA strand ends independent of homology, and homologous recombination, which requires DNA sequence homology (Cahill et al., 2006; Sonoda et al., 2006). Unlike *Saccharomyces cerevisiae*, which uses homologous recombination to repair double stranded breaks, most fungi preferentially use non-homologous end joining (Schiestl et al., 1994). Consequently during transformation, exogenous DNA is predominantly integrated randomly into the genome resulting in poor gene deletion or targeted plasmid integration frequencies, regardless of the presence of DNA sequence homology between the introduced construct and the recipient genome. An additional issue is that random ectopic integration can lead to position specific effects that may complicate the phenotypic analysis. The non-homologous end joining recombination pathway requires a DNA-dependent protein kinase catalytic subunit (DNA-PKcs), the Ku70/Ku80 heterodimer and the DNA ligase Lig4 (reviewed in Critchlow and Jackson 1998). Deletion of the fungal orthologs of Ku70, Ku80 and Lig4 has been shown to significantly reduce the frequency of non-homologous integration of incoming DNA fragments leading to dramatically improved gene deletion and targeted integration efficiency (Goins et al., 2006; Ishibashi et al., 2006; Krappmann et al., 2006; Meyer et al., 2007; Mizutani et al., 2008; Nayak et al., 2006; Ninomiya et al., 2004; Poggeler and Kuck, 2006; Takahashi et al., 2006a,b).

Disease causing fungi are of increasing economic and medical importance (van Burik and Magee, 2001; Gow et al., 2002; Hogan et al., 1996; Magee et al., 2003). *Penicillium marneffei* is an opportunistic pathogen of humans and has been developed as a model system for investigating the molecular mechanisms controlling morphogenesis and host-pathogen interactions. Typical of a number of pathogenic fungi, *P. marneffei* is capable of two modes of vegetative growth (known as dimorphism): a multicellular hyphal growth form at room temperature (25 °C) and a single-celled yeast growth form evident at 37 °C and in the human host that divides by fission (Andrianopoulos, 2002; Klein and Tebbets, 2007; Vanittanakom et al., 2006). *P. marneffei* is highly amenable to molecular genetic manipulation, the dimorphic switch is readily induced by changing the incubation temperature, and the entire genome has

* Corresponding author. Fax: +61 3 8344 5139.
E-mail address: alex.a@unimelb.edu.au (A. Andrianopoulos).
[1] These authors are equally contributed to this work.

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recently been sequenced, facilitating rapid gene identification. To facilitate gene characterization, exogenous DNA can be introduced into *P. marneffei* efficiently using polyethylene glycol-mediated DNA transformation of protoplasts, and a suite of selectable markers have been developed for this purpose (Borneman et al., 2001; Boyce et al., 2012). Due to strong bias towards non-homologous integration of incoming DNA, the ability to readily generate gene deletions is inefficient and also partially dependent on the target gene locus and the length of homologous sequence in the construct being introduced. Additional copies of the deletion construct are also frequently integrated ectopically at random locations throughout the genome. To improve this procedure we have eliminated components of the non-homologous end joining DNA repair pathway, encoded by the genes *pkuA* and *ligD*, thereby blocking DNA integration via this process, and examined the consequences. The data shows a dramatic improvement in the ability to generate gene deletion strains to assess gene function. Using PCR-based cloning, a pipeline has been developed to facilitate the rapid generation of gene deletion constructs mediated by *in vitro* recombination using the Gateway™ system, and subsequent efficient homologous recombination in a Δ*ligD* recipient strain.

2. Materials and methods

2.1. Molecular techniques

Plasmid DNA was isolated using the Wizard Plus SV DNA Purification System (Promega). Genomic DNA was prepared from frozen mycelia of *P. marneffei* as described previously (Borneman et al., 2001). Southern blots were prepared with Hybond N+ membrane (Amersham) using standard procedures (Sambrook et al., 1989). For screening of the *P. marneffei* genomic DNA lambda library, plaque lifts and the isolation of positive clones was performed according to the instructions of the λBlueSTAR vector system kit (Novagen). Hybridizations were performed with [α-^{32}P]-dATP-labelled DNA probes using standard methods (Sambrook et al., 1989).

2.2. Cloning and plasmid construction

Primers used in polymerase chain reactions (PCRs) to isolate fragments for cloning are listed in Table 1 and a description of the plasmids used in this study is provided in Table 2.

The *Aspergillus nidulans nkuA* (AN7753.2) (Nayak et al., 2006) was used as a heterologous probe to screen a *P. marneffei* λ-based genomic DNA library at low stringency (40% formamide, 2X SSC, 37 °C). Southern blot hybridization analysis and sequencing confirmed that clone pEG6376 contained the *P. marneffei* KU70 homolog *pkuA* (PMAA_037760). A *pkuA* gene deletion construct was generated where a 2316 bp *Sac*II/*Xba*I fragment from pEG6376 was cloned into pAB4626, containing the *A. nidulans pyrG* blaster cassette (Borneman et al., 2001). Subsequently, a 2546 bp *Xho*I/*Eco*RI fragment generated by PCR using the primers Y37 and Y38 was cloned into the *Xho*I/*Eco*RI sites to produce pKB6757. This construct was PCR amplified with primers I29 and H56 for transformation into *P. marneffei* strain SPM4.

A modified version of pDONR™221 containing the *A. nidulans pyrG* blaster cassette was constructed by amplifying the *attB1* and *attB2* sites as well as the kanamycin resistance encoding vector backbone of pDONR™221 by inverse PCR using the MM8 and MM9 primers. This blunt end fragment was then ligated to a 2.2 kb *Sma*I/*Eco*RV *AnpyrG* fragment from pAA4626 to generate pHW7711.

The *P. marneffei LIG4* ortholog *ligD* (PMAA_031390) was isolated by PCR using the primers MM60 and MM61 and cloned into the *Eco*RV site of pBluescript II SK$^+$ to give rise to pHB7676. The gene deletion construct pHB7677 was generated using the Gateway™ system. Using pHB7676 as a template, inverse PCR was performed with the primers MM62 and MM63 to isolate the 5′ and 3′ flanking regions of *ligD*. The *A. nidulans pyrG* blaster cassette was inserted into this plasmid by site-specific recombination using pHW7711 as the donor vector. This involved a BP clonase recombination reaction, performed according to the manufacturers' instructions (Invitrogen), using 30 ng of gel purified inverse PCR product. A linear fragment of pHB7677 was generated using PCR (MM60 and MM61) for transformation into *P. marneffei* strain SPM4.

The PMAA_068430 gene of *P. marneffei* was cloned by PCR using the primers BB63 and BB64 and cloned into pGEM-T Easy (Promega) to generate pTS7100. A deletion construct pHB7673 was generated using the modified Gateway™ system, whereby an inverse PCR product was amplified using the primers MM22 and MM23 to isolate the 5′ (1 kb) and 3′ (1.7 kb) flanking regions of PMAA_068430. The *A. nidulans pyrG* blaster cassette was inserted into this plasmid by site-specific recombination using pHW7711 as the donor vector. A linear fragment of pHB7673 was generated using PCR (MM22 and MM23) for transformation into *P. marneffei*.

Table 1
Oligonucleotides used in this study.

Name	Template	Sequence 5′–3′
Y37	PMAA_037760 (*pkuA*)	CTGAATTCTTGCTCTCGCCGCC
Y38	PMAA_037760	CTCTCGAGTCCCTGGCTGGTTG
MM60	PMAA_031390 (*ligD*)	GCTTAGTGGATGAACATTGG
MM61	PMAA_031390	GCATTGGTCTTTATCACAGC
MM62	PMAA_031390	GGGGACCCAGCTTTCTTGTACAAAGTGGTGT GAGATTGATGATAATG
MM63	PMAA_031390	GGGGAGCCTGCTTTTTTGTACAAACTTGTTG GAGATACTGAAAAGCC
I29	pBluescript II SK$^+$	GTAAAACGACGGCCAGT
H56	pBluescript II SK$^+$	GGAAACAGCTATGACCATG
MM8	pDONR™221-*att*P2	TGAGGACAATAGCGAGTAGG
MM9	pDONR™221-*att*P1	GATTCAACAGGGACACCAGG
BB63	PMAA_068430	ATGGCATTGTCCTCCGTC
BB64	PMAA_068430	TGGCGTCTGAATAATCCG
MM22	PMAA_068430	GGGGACCCAGCTTTCTTGTACAAAGTG GTTCTGGCGAACATTGACTGG
MM23	PMAA_068430	GGGGAGCCTGCTTTTTTGTACAAACTT GTTTTGAGGTGCCGATATGC

Table 2
Plasmids used in this study.

Plasmid	Description
pEG6376	Clone containing *P. marneffei pkuA* (PMAA_037760)
pKB6757	*pkuA* gene deletion construct containing the *AnpyrG* selectable marker
pHB7676	Clone containing *P. marneffei ligD* (PMAA_031390)
pHB7677	*ligD* gene deletion construct containing the *AnpyrG* selectable marker
pAB4626	*A. nidulans pyrG* gene, flanked by CAT repeats (Borneman et al., 2001).
pHW7711	Modified pDONR™221 containing the *AnpyrG* selectable marker
pTS7100	Clone containing PMAA_068430
pHB7673	PMAA_068430 deletion construct containing the *AnpyrG* selectable marker
pDONR™221	Gateway™ plasmid containing the *att*P1 and *att*P2 sites and kanamycin resistance

2.3. Fungal strains and media

The *P. marneffei* strains used in this study are listed in Table 3. The isolation and transformation of *P. marneffei* protoplasts was