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Strategies for the molecular genetic manipulation and visualization of the human fungal pathogen Penicillium marneffei

Abstract

P. marneffei has been established as an experimentally amenable system to study morphogenesis and pathogenicity. This paper describes the development of a number of tools, including numerous selectable markers, to expand the ease with which it can be genetically manipulated. Combined with strains engineered for homologous recombination of exogenous DNA, these tools facilitate efficient molecular genetic studies.

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Strategies for the molecular genetic manipulation and visualization of the human fungal pathogen *Penicillium marneffei*

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P. marneffei has been established as an experimentally amenable system to study morphogenesis and pathogenicity. This paper describes the development of a number of tools, including numerous selectable markers, to expand the ease with which it can be genetically manipulated. Combined with strains engineered for homologous recombination of exogenous DNA, these tools facilitate efficient molecular genetic studies.

Introduction

To facilitate molecular genetic analysis of gene function in *P. marneffei*, an important opportunistic pathogen of humans, an efficient DNA-mediated transformation protocol was developed using exogenous DNA and polyethylene glycol-mediated protoplast fusion (Borneman *et al*. 2001). Spontaneous mutants were also derived from the *P. marneffei* type strain FRR2161 (ATCC18224) by selection on the toxic compounds 5-fluoroorotic acid (5-FOA) and chlorate to generate *pyrG* (orotidine 5'-monophosphate decarboxylase) auxotrophic and *niaD* (nitrate reductase) utilization mutants, respectively (Table 1 and 2)(Borneman *et al*. 2001). Combined with the dominant selectable marker of bleomycin/phleomycin resistance and the ability to recycle the *pyrG* marker (Borneman *et al*. 2001), complex genetically modified strains can be created for analysis. Exogenous DNA introduced during transformation preferentially integrates into the genome of *P. marneffei* by non-homologous integration, however, strains defective in the non-homologous end-joining machinery have recently been developed that result in highly efficient homologous integration (Table 1)(Bugeja *et al*, 2012). This study describes the development of additional auxotrophic and dominant selectable markers to broaden the options for selection of transformants containing introduced DNA in the type strain of *P. marneffei* or into clinical isolates (Table 2). In addition, a number of constructs have been developed for targeted integration at specific loci and for the rapid generation of gene deletion constructs using the previously described Gateway™ cloning system (Bugeja *et al*, 2012). Lastly, tools for the microscopic visualization of *P. marneffei* mutants generated by these techniques are described.

The *riboB* and *pyroA* selectable markers in *P. marneffei*

In order to develop additional selectable markers for use with auxotrophic *P. marneffei* strains, the *riboB* and *pyroA* genes encoding a GTP cyclohydrolase and 5'-phosphate synthase required for riboflavin and pyridoxine biosynthesis, respectively, were cloned and deleted (Oakley *et al*. 1987b; Osmani *et al*. 1999). The *P. marneffei riboB* gene was PCR amplified (primers AA18 and AA19) and cloned into pBluescript II SK^+ (Stratagene)(pAA7329, Table 3 and 4). A split marker construct was generated by overlap PCR to facilitate deletion of the *riboB* locus using the *A. nidulans pyrG* blaster cassette (pHB7131 and pHB7132; Tables 3 and 4). A *riboB* gene deletion strain, in which the entire coding region has been replaced with the *A. nidulans pyrG* blaster cassette, was been generated in the SPM4 (G779) and Δ*ligD pyrG*$^-$ (G829) strains (Table 1). *pyrG*$^-$ derivatives (G780 and G890) have also been isolated as 5-FOA resistant sectors which have lost the *A. nidulans pyrG* gene (Borneman *et al*. 2001). *P. marneffei* Δ*riboB* strains require supplementation with 5 µg mL^{-1} riboflavin and must be grown in the dark to prevent photolytic breakdown of riboflavin (Table 2).

Table 1. *P. marneffei* strains used in this study

Strain	Genotype	Origin
FRR2161	Wild type	Bamboo rat (*Rhizomys sinensis*) isolate (Supplied by J. Pitt, CSIRO Food Industries, Sydney, Australia). Also available from the American Type Culture Collection as ATCC18224
G146 (SPM3)	*niaD1*	(Borneman *et al*. 2001)
G147 (SPM4)	*niaD1 pyrG1*	(Borneman *et al*. 2001)
G809	*niaD1 pyrG1* Δ*ligD::AnpyrG*$^+$	(Bugeja *et al*, 2012)
G816	*niaD1 pyrG1* Δ*ligD*	(Bugeja *et al*, 2012)
G779	*niaD1 pyrG1* Δ*riboB::AnpyrG*	This study
G829	*niaD1 pyrG1* Δ*ligD riboB::AnpyrG*	This study
G780	*niaD1 pyrG1* Δ*riboB*	This study
G890	*niaD1 pyrG1* Δ*ligD riboB*	This study
G830	*niaD1 pyrG1* Δ*ligD pyroA::AnpyrG*	This study
G908	*niaD1 pyrG1* Δ*ligD pyroA*	This study
G487	*niaD1 pyrG1 areA*$^{\Delta DBD}$	This study
G831	*niaD1 pyrG*$^+$ *HI::mCherry* Δ*ligD*	This study

The *P. marneffei pyroA* gene was amplified (primers AA20 and AA21), cloned (pAA7331) and a split marker gene deletion construct was generated using overlap PCR (pHB7129 and pHB7130; Table 3 and 4). A *pyroA* gene deletion strain, in which the entire coding region has been replaced with the *A. nidulans pyrG* blaster cassette, was generated in the Δ*ligD pyrG*$^{-}$ strain (G830) and a *pyrG*$^{-}$ derivative (G908) has also been obtained as described above (Borneman *et al*. 2001). Similar to *A. nidulans*, the *P. marneffei* Δ*pyroA* strain requires supplementation with 1µg mL^{-1} pyridoxine (Table 2). Despite supplementation, it has been observed that this strain grows slower than *pyroA*$^{+}$ strains and has slightly reduced conidiation at 25°C.

Table 2. *P. marneffei* selectable marker systems

Selectable marker	Recipient strain	Phenotype of recipient strain	Selection regime
pyrG	*pyrG1*	Uracil auxotroph (requires 10 mM uracil), 5'FOA resistant	Positive: No added uracil Negative: 1 mg mL^{-1} 5-FOA + 10 mM uracil
niaD	*niaD1*	Nitrate utilization defect, Chlorate resistant	Positive: 10 mM nitrate as the sole nitrogen source Negative: 10 mM chlorate
riboB	Δ*riboB*	Riboflavin auxotroph (requires 5 µg mL^{-1} riboflavin & growth in dark)	No added riboflavin
pyroA	Δ*pyroA*	Pyridoxine auxotroph (requires 1 µg mL^{-1} pyridoxine)	No added pyridoxine
barA	Any	N/A	25-50 µg mL^{-1} of PPT
ptrA	Any	N/A	0.1-0.2 mg mL^{-1} of pyrithiamine hydrobromide
barA/hv-tk	Any	N/A	Positive: 25-50 µg mL^{-1} of PPT Negative: 5 µM FDU
areA	*areA*$^{\Delta DBD}$	Reduced growth on nitrogen sources other than ammonium	10 mM nitrite as the sole nitrogen source
wA	Any	Green conidiation	Screen for white conidiation phenotype

Employing dominant selectable markers for transformation of *P. marneffei*

Since it was first developed as a dominant selectable marker in *N. crassa,* L-phosphinothricin

(PPT), also known as glufosinate ammonium, resistance has been used in many fungi (Ahuja and Punekar 2008; Avalos *et al*. 1989). L-phosphinothricin inhibits glutamine synthetase, required for ammonium assimilation, by occupying the substrate (glutamate) pocket (Gill and Eisenberg 2001). The *Aspergillus oryzae barA* gene encodes PPT resistance and can be used in *P. marneffei* for transformation (Table 5). Similar to *A. nidulans* and *N. crassa,* selection of *P. marneffei* PPT resistant transformants requires approximately 25-50 µg mL^{-1} of PPT (Table 2)(Ahuja and Punekar 2008).

Table 3. Oligonucleotides used in this study

Name	Template	Sequence 5' – 3'
AA18	*riboB*	CAGCGTGTCGAGTTCAGG
AA19	*riboB*	TCTCTCTCCATCAGCGGC
CC63	*riboB*	AAGGGTGAACACTATCCTTGCGGAGCGATGT
CC64	*riboB*	CCGTACATCGCTCCGCAAGGATAGTGTTCAC
CC65	*riboB*	GATGAGTGGCAGGGGGCTGCAGAAAGGTATA
CC66	*riboB*	CTCCTTATACCTTTCTGCAGCCCCCTGCCAC
QQ11	*riboB*	TGACCATGATTACGCCAAGC
QQ12	*riboB*	TGAATTCGAGCTCGGTACCC
AA20	*pyroA*	TCAAGCCGTTACTGTCCA
AA21	*pyroA*	GACTTTGAACGGCACATT
CC59	*pyroA*	AAGGGTGAACACTATCCATTCTCGGATGCCATTGC
CC60	*pyroA*	GCAATGGCATCCGAGAATGGATAGTGTTCACCCTT
CC61	*pyroA*	GATGAGTGGCAGGGGGCTATTGGCGTTAAGCGGTGG
CC62	*pyroA*	CCACCGCTTAACGCCAATAGCCCCCTGCCACTCATC
NN61	*ptrA*	TTACGGGATCCCATTGGTAA
NN62	*ptrA*	AAGACAAATCCGGTTCATGC
AA52	*pyrG*	CTTATCGGGCCGGAGCA
AA53	*pyrG*	ATCCTCGCTCTCCTCTTTCT
HH81	*pyrG*	GAGTTTGTCGTGCTATCCG
II01	*pyrG*	GTCTTGCTTACGGTACTCC
QQ39	*pyrG*	CGCAAACTATTCGCTACTG
KK77	*niaD*	CGAAACCAAGACGAGAGGC
KK78	*niaD*	TTGGATGGGAAGAGAGCG
II08	*wA*	AATCTAGAGCCTATGCCTATGTCTTC
II09	*wA*	AAGCGGCCGCGAATGCGACAGGCGTATC
HH45	*wA*	GTCATGCAGGAAAGGGTCAT
HH46	*wA*	GCACCGGTCGATACTTGAAT
LL9	*mCherry*	ACTAGTGCCGGTGCGCCCGGG
LL10	*mCherry*	TCTAGACCAGCATCTGATGTCCTGGT
LL11	*H1*	GGATCCCCCTTGACAAAACCGCAGAG
LL12	*H1*	ACTAGTGGCCTTCTTGGGTGTGGCCT
MM8	pDONR^{TM}221-*att*P2	TGAGGACAATAGCGAGTAGG
MM9	pDONR^{TM}221-*att*P1	TGAGGACAATAGCGAGTAGG

Table 4. Plasmids used in this study

Plasmid	Name	Description
pPL3	*An riboB*	*A. nidulans riboB* gene (Oakley *et al*. 1987b)
pAA7329	*riboB*	*riboB* gene amplified using AA18 and AA19 and cloned into the *EcoRV* site of pBluescript SK^+
pHB7131	5'Δ*riboB::pyrG*	5' *riboB* fused to the 5' half of *pyrG*. Overlap PCR using AA18, CC63, CC64 and AA52. Amplify using AA18 and AA52.
pHB7132	3'Δ*riboB::pyrG*	3' *riboB* fused to the 3' half of *pyrG*. Overlap PCR using CC65, AA19, CC66 and AA53. Amplify using AA19 and AA53.
pAA7331	*pyroA*	*pyroA* gene amplified using AA20 and AA21 and cloned into the *EcoRV* site of pBluescript II SK^+ (Osmani *et al*. 1999).
pHB7129	5'Δ*pyroA::pyrG*	5' *pyrA* fused to the 5' half of *pyrG*. Overlap PCR using AA21, CC59, CC60 and AA52. Amplify using AA21 and AA52.
pHB7130	3'Δ*pyroA::pyrG*	3' *pyro*A fused to the 3' half of *pyrG*. Overlap PCR using AA20, CC61, CC62 and AA53. Amplify using AA20 and AA53.
pAA5962	MT1612	*barA*
pTW7703	*ptrA* SK^+	*A. oryzae ptrA* PCR amplified from PTRII using primers NN61 and NN62 and cloned into the *SmaI* site of pBluescript II SK^+.
pTW7704	*ptrA* SK^+	Same as pTW7703 with insert in opposite orientation.
pTW7705	*ptrA* SK^+ backbone	*A. oryzae ptrA* PCR amplified from PTRII using primers NN61 and NN62 and blunt cloned into the *SspI* site of the pBluescript II SK^+ backbone.
pDG18	$trpC_{(p)}$*::hv-tk::*$trpC_{(t)}$	Contains the *hv-tk* gene encoding herpes simplex virus thymidine kinase under the control of the *A. nidulans trpC* promoter and terminator (Gardiner and Howlett 2004).
pKB7410	*pyrG* (2161) pGTE	*pyrG* cloned from strain FRR2161 ($pyrG^+$) using primers HH81 and II01 and cloned into pGEM-T Easy.
pKB7411	*pyrG* (SPM4) pGTE	*pyrG* cloned from strain SPM4 ($pyrG^-$) using primers HH81 and II01 and cloned into pGEM-T Easy.
pLS7804	*pyrG* targeting	*P. marneffei pyrG* (missing 60 bp of coding region) amplified with QQ39 and II01, digested with *EcoICRI* and cloned into the *SspI* sites of the pBluescript II SK^+ backbone.
pHB7612	*P. marneffei niaD*	*niaD* was PCR amplified using KK77 and KK78 and cloned into pBluescript II SK^+.
pHB7613	*niaD* targeting	*EcoRI* fragment deletion to remove the 5' 546 bp of *niaD* to allow targeted integration.
pHB7615	*niaD* targeting SK^+ backbone	2.8 kb *EcoRI/HindIII* fragment from pHB7613 was made blunt ended with Klenow and cloned into the *SspI* sites in the pBluescript II SK+ backbone.
pHB6104	*areA*	Full length *areA* gene (Bugeja *et al*, 2012b)
pHB7186	*areA* targeting	2.6 kb *EcoICRI/EcoRV* fragment from pHB6104 cloned into the *SspI* sites of the pBluescript II SK^+ backbone.
pSJ7351	*wA* pGTE	8.5 kb coding region of *wA* amplified using HH45 and HH46 and cloned into pGEM-T Easy.
pSB7364	*wA* targeting ($pyrG^+$)	A *NotI* fragment of a 1.7 kb *wA* PCR (amplified from pSB7351 using II08 and II09) was cloned into pALX223.
pDONR^{TM}221	Gateway cloning vector	Invitrogen
pHW7711	pDONR^{TM}221::*pyrG*	Bugeja *et al*, 2012
pHW7856, pHW7857.	pDONR^{TM}221 ::*pyroA*	Inverse PCR of pDONR^{TM}221 with the MM8 and MM9 primers ligated to the *pyroA* fragment from pAA7331. *pyroA* selectable marker available in both orientations.

pHW7771, pHW7772	pDONR^{TM}221 ::*barA*	Inverse PCR of pDONR^{TM}221 with the MM8 and MM9 primers ligated to the 1443 bp *EcoRV barA* fragment from pAA5962. *barA* selectable marker available in both orientations.
pHW7773, pHW7774	pDONR^{TM}221 ::*riboB*	Inverse PCR of pDONR^{TM}221 with the MM8 and MM9 primers ligated to a 2663 bp *AnriboB* PCR product from pPL3 amplified using the primers QQ11 and QQ12. *AnriboB* selectable marker available in both orientations.
pMP7742	pDONR^{TM}221 ::*ptrA*	Inverse PCR of pDONR^{TM}221 with the MM8 and MM9 primers ligated to an *EcoICRI/EcoRV ptrA* fragment from pTW7703.
pMP7601	mCherry pGTE	*mCherry* PCR amplified from LO1945 (Supplied by B. Oakey) using LL09 and LL10 and cloned into pGEM-T Easy.
pMP7602	*H1* pGTE	Histone *H1* PCR amplified from FRR2161 genomic DNA with LL11 and LL12, which adds a 5' *BamH1* site and a 3' *SpeI* site, removes the stop codon and contains 1.7 kb of promoter.
pMP7605	*H1*(p)::*H1*::mCherry *pyrG*	*BamHI/SpeI* pMP7602 ligated to *BamHI/SpeI* pALX223. This clone was digested with *SpeI/XbaI* and ligated to *SpeI/XbaI* fragment from pMP7601.

Table 5. Plasmids for use in the selectable marker systems

Selectable marker	Plasmid	Description	Reference
pyrG	pAA4707	*A. nidulans pyrG*	(Oakley *et al*. 1987a)
	pAB4626	*A. nidulans pyrG* blaster cassette	(Borneman *et al*. 2001)
	pHW7711	Gateway^{TM} plasmid containing *A. nidulans pyrG* blaster cassette flanked by *att*P sites	(Bugeja *et al*, 2012)
	pSB7364	*wA* targeting construct contains *A. nidulans pyrG* blaster cassette	This study
	pLS7804	*pyrG* targeting construct	This study
niaD	pSTA14	*A. oryzae niaD*	(Unkles *et al*. 1989)
	pHB7613, pHB7615	*niaD* targeting construct	This study
riboB	pPL3	*A. nidulans riboB* gene	(Oakley *et al*. 1987b)
	pHW7773, pHW7774	Gateway^{TM} plasmid containing *A. nidulans riboB* gene flanked by *att*P sites	This study
pyroA	p14	*A. nidulans pyroA* gene	(Osmani *et al*. 1999)
barA	pSM6287	*A. oryzae barA* gene	(Nayak *et al*. 2006)
	pHW7771, pHW7772	Gateway^{TM} plasmid containing *A. oryzae barA* gene flanked by *att*P sites	This study
ptrA	pTW7703-7705	*A. oryzae ptrA* gene	This study
	pMP7742	Gateway^{TM} plasmid containing *A. oryzae ptrA* gene flanked by *att*P sites	This study
barA/hv-tk	pHW7591	Plasmid containing *A. oryzae barA* for positive selection and *hv-tk* for negative selection	This study
areA	pHB7186	*areA* targeting construct	This study

Pyrithiamine resistance was first developed as a dominant selectable marker in *A. oryzae* and has since been shown to be effective in a number of filamentous fungi (Kubodera *et al*. 2000; Kubodera *et al*. 2002). Pyrithiamine is a thiamine analogue, that binds to thiamine pyrophosphate

riboswitches, small RNA elements that bind thiamine pyrophosphate to regulate the expression of genes required for the biosynthesis and transport of thiamine, an essential cofactor (Sudarsan *et al*. 2005). Pyrithiamine resistance can also be utilized as a dominant selectable marker for transformation in *P. marneffei* as *ptrA* containing plasmids (Table 5) confer resistance to pyrithiamine, with transformants selected on 0.1-0.2 mg mL^{-1} of pyrithiamine hydrobromide (Sigma)(Table 2). Occasionally, a low number of spontaneously resistant colonies can arise during transformation without the addition of exogenous DNA.

Selectable marker plasmids facilitating positive/negative selection

In circumstances where constructs may be transiently required, certain selectable markers may also be used for negative selection of constructs. This has been demonstrated previously for recycling of the *pyrG* selectable marker (Borneman *et al*. 2001). Both the *pyrG* and *niaD* genes facilitate negative selection, in addition to positive selection, since mutations cause resistance to the toxic compounds 5-fluoroorotic acid (5-FOA) or chlorate, respectively (Table 2). A new construct has been developed which can also be used for both positive and negative selection (pHW7709, Table 5). This construct contains the previously described *barA* gene, used as a positive selectable marker and the 'dominant' Herpes Simplex virus thymidine kinase encoding gene (*hv-tk*) as a negative selectable marker, which confers sensitivity to the toxic thymidine analogue 5-fluorodeoxyuridine (FDU) (Table 2 and 5) (Sachs *et al*, 1997; Ahuja and Punekar 2008; Gardiner and Howlett 2004; Gill and Eisenberg 2001). To counter-select against *hv-tk*, strains are plated on medium containing 5 µM 5-fluorodeoxyuridine (FDU)(Sigma). Southern blot hybridisation analysis should be used to confirm loss of the constructs containing the negative selectable markers, as opposed to point mutations that could result in the same phenotype, albeit at a lower frequency.

Targeted integration of plasmids

Targeted integration of constructs at specific loci offers many advantages over non-specific ectopic integration by overcoming possible copy number and position effects. A series of targeting constructs were generated to allow for the integration of plasmids at known genomic locations, including *pyrG, areA, niaD* and *wA* (Table 6). When a recipient strain is transformed with the appropriate targeting plasmid, a single homologous recombination event leads to the integration of the plasmid thus restoring gene function (*pyrG, areA* and *niaD*) or resulting in a visible phenotype (*wA*) (Figure 1). Ectopic integration will not result in these selected phenotypes. The plasmids used for targeted integration at *pyrG*, *niaD* or *areA* all contain a portion of the selectable marker cloned into the *SspI* sites of the pBluescript II SK^+ backbone to permit blue/white screening to be used when additional DNA fragments are cloned into the polylinker (Table 4).

The $pyrG^-$ and $niaD^-$ mutations in strain SPM4 (G147) were identified by sequencing of the PCR products spanning these genes (Table 6). Plasmids for *pyrG* and *niaD* targeting lack the start codon of these genes but contain the regions that span the loss-of-function point mutations. Thus, a single crossover event between the start codon and the mutated region of the genomic allele leads to integration of the plasmid and restoration of gene function, that is the ability to grow in the absence of uracil or on nitrate as a sole nitrogen source (Table 2).

Table 6. *P. marneffei* gene targeting regimes

Gene targeted	Recipient strain	Nature of mutation in recipient strain	Region of gene sequence included for targeting
pyrG	*pyrG*$^{-}$	L155V and I156T in the decarboxylase domain	Contains all but first 60 bp of *pyrG* coding sequence (pLS7804)
wA	Any with green conidiation[a]	N/A	Internal portion (+19 to +932) of the *wA* coding sequence (pSB7364)
areA	*areA*$^{\Delta DBD}$	Deletion of DNA binding domain	Contains 3' half of *areA* including DNA binding domain but lacks the START codon (pHB7186)
niaD	*niaD*$^{-}$	N293K and K513N	Contains all but first 546 bp of *niaD* coding sequence (pHB7615)

[a] Requires additional selection. Transformants with integration at *wA* locus will display a white conidiation phenotype

Additionally, the *areA* gene, encoding the GATA transcription factor required for growth on non-preferred nitrogen sources, was also developed as a locus for targeted integration (Bugeja *et al*, 2012b). This locus displays a high-rate of homologous integration and has been modified such that the DNA-binding domain has been deleted (*areA*$^{\Delta DBD}$), resulting in loss of gene function, yet the majority of the coding region is still intact. A plasmid containing the 3' half of *areA*, including the DNA-binding domain is used for targeted integration (Table 6). When *areA*$^{\Delta DBD}$ strains are transformed with the *areA* targeting plasmid, a single crossover event integrates the plasmid at *areA* thus restoring the ability to utilise non-preferred nitrogen sources (Table 2, Figure 1). Screening integration events by Southern blot analysis is crucial as a double crossover or gene conversion events are also possible and will lead to *areA*$^{+}$ without integrating the entire plasmid (Figure 1).

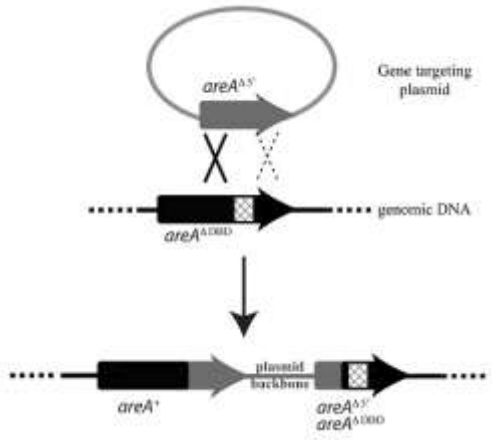

Figure 1. Targeting to the *areA* locus in the *areA*$^{\Delta DBD}$ strain

The plasmid for gene targeting to the *areA* locus contains a 5' truncated allele of the *areA* gene (grey shading). When *areA*$^{\Delta DBD}$ strains are transformed with the *areA* targeting plasmid, a single recombination event will integrate the plasmid into the genomic region containing the *areA*$^{\Delta DBD}$ locus (black shading) via a single crossover event (solid cross) in the homologous region 5' of the DBD deletion (hatched box). This will regenerate a wild-type *areA*$^{+}$ gene thus restoring the ability to utilise nitrite as the sole nitrogen source, in addition to, a copy of *areA* that contains both the 5' truncation and the DBD deletion flanking the integrated vector sequences. The dashed cross depicts an alternate homologous recombination event that may also occur to regenerate a wild-type *areA*$^{+}$ gene without integration of the vector sequences.

The polyketide synthase encoding gene, *wA (pksP)*, is required for DHN melanin synthesis during asexual development, resulting in pigmentation of the asexual spores (conidia), which can be detected visually at the colony level (Mayorga and Timberlake 1990; Mayorga and

Timberlake 1992) (Table 6). The *P. marneffei wA* targeting construct contains an internal portion of the *wA* coding sequence, in addition to the *A. nidulans pyrG* selectable marker (Table 4). Transformants of *pyrG*$^{-}$ recipient strains are selected for uracil prototrophy (*pyrG*$^{+}$), and secondarily screened for a white conidial phenotype indicating that the construct has integrated via a single cross over event at the *wA* locus resulting in gene disruption (Table 6). It should be noted that *A. fumigatus pksP* (orthologous to *wA*) mutants display decreased virulence in a mouse model of aspergillosis and *P. marneffei* disruption mutants also have attenuated virulence (Jahn *et al*. 2000; Jahn *et al*. 2002; Langfelder *et al*. 1998; Woo *et al*. 2010). Therefore *P. marneffei wA* targeting should not be utilized in strains that will be subsequently tested for virulence attributes.

Selectable markers available for the generation of deletion constructs using a Gateway™ cloning system

A pipeline for the cloning and functional characterization of genes in *P. marneffei* utilizing a Gateway™ cloning system to facilitate the rapid generation of gene deletion constructs has been developed (Bugeja *et al*, 2012). This approach uses a PCR and recombination based system where the flanking regions of genes to be deleted are amplified by inverse PCR to incorporate *att*B recognition sequences, which facilitates integration of a selectable marker by *in vitro* recombination with corresponding *att*P sequences. Gateway™ plasmids containing *A. nidulans pyrG* (pHW7711; Figure 2A), *riboB* (pHW7771 and pHW7772; Figure 2B), *pyroA* (pHW7856 and pHW7857, Figure 2C) and *A. oryzae barA* (pHW7773 and pHW7774; Figure 2D) and *ptrA* (pMP7742; Figure 2E) have been constructed to allow the rapid generation of deletion constructs (Table 4). These plasmids (which confer kanamycin resistance in *E.coli*) have been engineered to contain the selectable marker gene flanked by *att*P1 and *att*P2 sites. The nature of the selectable markers means that these constructs can also be used in fungi other than *P. marneffei*.

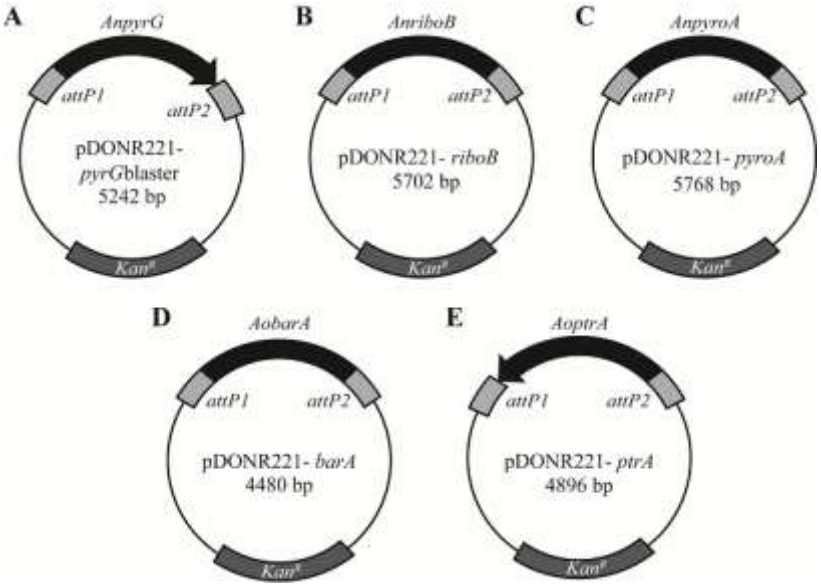

Figure 2. Plasmids for the generation of deletion constructs using a Gateway™ cloning system
Plasmids contain kanamycin resistance (Kan^R) and a selectable marker flanked by *att*P1 and *att*P2 sites. Selectable markers: A. *A. nidulans pyrG* (pHW7711), B. *riboB* (pHW7771), C. *pyroA* (pHW7856) and D. *A. oryzae barA* (pHW7773) and E. *ptrA* (pMP7742). Plasmids containing *riboA, pyroA* and *barA* are available with selectable markers in both orientations.

Tools for microscopic visualization of *P. marneffei*

P. marneffei mutants generated using the molecular tools described above are commonly characterized for morphological defects microscopically by observing hyphae, conidiophores and yeast cells. A number of cellular stains and fluorescent fusion proteins can be used to allow microscopic visualization of the cell membrane or wall and nuclei. For microscopic visualization, *P. marneffei* strains can be grown as liquid cultures in shake flasks or microtitre plates, or on slides covered with a thin layer of solid medium with one end resting in liquid medium (Borneman *et al*. 2000). When required cells can be fixed by soaking them in a solution of 4% para-n-formaldehyde in PME (50 mM PIPES pH 6.7, 1 mM $MgSO_4$, 20 mM EGTA) for 30 minutes, followed by two 5 minute PME washes.

P. marneffei cell membranes can be visualized using the lipophylic membrane dye FM4-64 (Invitrogen) and is performed by immersing unfixed slides in 25 μM FM4-64 (suspended in water) for 15 minutes at room temperature, washing and mounting in water. In the wild type, FM4-64 staining is observed around the cell periphery, surrounding vesicles at the hyphal apex, as a crescent at the presumptive Spitzenkorper and as transverse membranes partitioning the hyphae into separate cellular compartments at septation sites (Figure 3A and B). Membrane sterols can be visualized using the fluorescent polyene macrolide stain, filipin, which specifically intercalates into sterol-rich membranes (Van Leeuwen *et al*. 2008). Sterol staining is performed by immersing unfixed cells in 5 mL of 25 μg mL^{-1} filipin (stock 1 mg mL^{-1} in DMSO, Sigma) for 5 minutes, followed by washing with liquid medium and visualization under UV. In wild type, ergosterol staining is observed concentrated at the hyphal apex, at the plasma membrane including at septa and as spots along the length of the hyphae (Figure 3C and D).

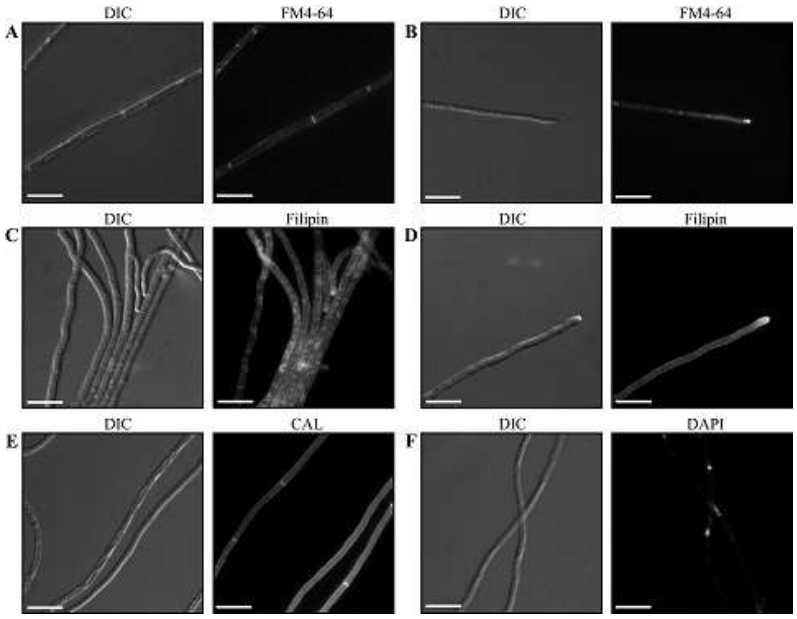

Figure 3. Microscopic visualization of *P. marneffei*

Wild type *P. marneffei* grown for 3 days at 25°C and stained with FM4-64 (A-B), Filipin (C-D), Calcofluor white (CAL)(E) or DAPI (F). Images were captured using differential interference contrast (DIC) or with epifluorescence to observe fluorescent stains. Scale bars, 20 μm.

Fluorescent brightener 28 (calcofluor white, Sigma) and FITC conjugated lectin (wheat germ agglutinin, WGA, Molecular Probes) can be used to visualize cell walls under UV (Figure 3E). Calcofluor is a non-specific fluorochrome which binds both cellulose and chitin in fungal cell walls, whereas, WGA specifically detects glycoproteins containing ß(1-4)-N-acetyl-D-glucosamine. For calcofluor white staining, 1 µL of a 1 µg μL^{-1} calcofluor white solution (suspended in water) is added directly to 5 µL of Tween 80 on the microscope coverslip prior to mounting. A modified protocol for WGA staining can be performed on live or fixed cells (Robin *et al*. 1986). Prior to staining, slides are incubated for 5 minutes in PME, 15 minutes in PME with 1 µg μL^{-1} BSA and then washed in PME. A 5 µL drop of a 5 µg μL^{-1} WGA solution (suspended in water) is added to the coverslip and slides are incubated in the dark for 30 minutes, before being washed with PME and mounted.

Under UV light nuclei can be observed in fixed *P. marneffei* cells stained with either 4,6-diamidino-2.phenylindole (DAPI) or Hoescht 33342 (Figure 3F). 1 µL of a 1 µg μL^{-1} solution of either stain (suspended in water) is added directly to 5 µL of Tween 80 on the coverslip prior to mounting. Nuclei can also be visualized using the *HI::mCherry* construct (pMP7605), which contains a fusion between the Histone HI and the mCherry fluorescent protein encoding gene (Table 4). A Δ*ligD pyrG*$^{-}$ *HI::mCherry* strain is also available as a transformation recipient strain (Table 2).

Combined with strains engineered for homologous recombination of exogenous DNA, the constructs for ectopic or site-specific integration and rapid generation of gene deletion constructs described in this study will greatly facilitate rapid and efficient analysis of gene function in *P. marneffei* and are available through the Fungal Genetics Stock Center (FGSC).

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