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Improved techniques for endogenous epitope tagging and gene deletion in *Toxoplasma gondii*

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ABSTRACT

Toxoplasma gondii is an excellent model organism for studies on the biology of the Apicomplexa due to its ease of in vitro cultivation and genetic manipulation. Large-scale reverse genetic studies in *T. gondii* have, however, been difficult due to the low frequency of homologous recombination. Efforts to ensure homologous recombination have necessitated engineering long flanking regions in the targeting construct. This requirement makes it difficult to engineer chromosomally targeted epitope tags or gene knock out constructs only by restriction enzyme mediated cloning steps. To address this issue we employed multisite Gateway® recombination techniques to generate chromosomal gene manipulation targeting constructs. Incorporation of 1.5 to 2.0 kb flanking homologous sequences in PCR generated targeting constructs resulted in 90% homologous recombination events in wild type *T. gondii* (RH strain) as determined by epitope tagging and target gene deletion experiments. Furthermore, we report that split marker constructs were equally efficient for targeted gene disruptions using the *T. gondii UPRT* gene locus as a test case. The methods described in this paper represent an improved strategy for efficient epitope tagging and gene disruptions in *T. gondii*.

1. Introduction

Toxoplasma gondii is an obligate intracellular protozoon parasite transmitted by ingestion of either food, containing bradyzoites or oocysts, or water, containing oocysts. It has a broad host range and geographical distribution with estimates that infection is present in at least 60 million people in the United States. *T. gondii* causes symptomatic infections in both immune competent and immune compromised individuals. The most commonly recognized infections are encephalitis in patients with HIV infection and chorioretinitis in the setting of congenital infection. In addition to its clinical significance, *T. gondii* is an excellent model organism for studies on the biology of the Apicomplexa due to its ability to be grown in tissue culture, the well developed genetic manipulation techniques for this organism (Kim and Weiss, 2004) and the available of extensive genome sequence data (Gajria et al., 2008).

Functional analysis of genes identified in genome sequencing projects is one of the prime research areas of the "post-genomic era". Epitope tagging has proven to be an important tool for the analysis of protein function, protein interaction and sub cellular distribution. These enable researchers to perform quickly experiments that previously were only feasible with the production of monoclonal or polyclonal antibodies. In addition, antibody production can be expensive, time consuming, yielding reagents of variable quality requiring extensive purification and characterization. In *T. gondii* plasmid based expression of epitope tagged proteins has been employed in several studies (Binder and Kim, 2004; Binder et al., 2008; Stedman et al., 2003; Striepen et al., 1998), but expression of tagged genes from a plasmid can cause artifacts due to the lack of regulated expression of the gene of interest (as would be seen at the endogenous gene locus).

Another powerful genetic strategy is gene deletion which can be performed on a genome wide scale as reported in the yeast *Saccharomyces cerevisiae* (Mnaimneh et al., 2004; Winzeler et al., 1999). *T. gondii*, being haploid is amenable for gene knockout studies and the essentiality of several *T. gondii* genes has been tested using this genetic approach (Binder et al., 2008; Donald and Roos, 1995, 1998; Zhang et al., 1999). Generation of gene specific knock out constructs often involves several laborious time consuming cloning steps and is not scalable. In bacteria and the yeast *S. cerevisiae* epitope tagging and gene deletion are of special interest because both of these processes are achievable at chromosomal loci by a simple PCR based strategy (Knop et al., 1999; Wach et al., 1997). Therefore, these

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procedures have become routine even on a genome wide scale (Ghaemmaghami et al., 2003; Huh et al., 2003). Gene replacement or modification requires homologous recombination at the target gene locus. In yeast due to the high frequency of homologous recombination only about 50 bp of homologous DNA sequences are sufficient for targeted gene manipulation (Wach et al., 1994).

The relative frequency of the homologous versus non homologous random recombination depends on the size of the flanking homology regions used in the engineered construct used for transfection. In organisms with a poor frequency of homologous recombination, including *T. gondii*, a large region of identical sequences is required in the targeting construct to induce homologous recombination. Recently, *T. gondii* strains lacking the *Ku80* protein which is required for the non homologous end joining of double strand DNA breaks, have been engineered (Fox et al., 2009; Huynh and Carruthers, 2009). Deletion of the Ku80 gene in various organisms has significantly enhanced homologous recombination, thereby requiring smaller regions for successful targeting (Ninomiya et al., 2004; Choquer et al., 2008; Goins et al., 2006). Absence of Ku80 protein in *T. gondii* led to a substantial increase in the efficiency of homologous recombination, by decreasing the chance of random integration. For gene deletion studies, DNA flanks of 500 to 1000 bp were sufficient for homologous recombination (Huynh and Carruthers, 2009).

Ku80 knock out (Δ*ku80*) strains were found to exhibit similar growth rate and virulence as compared to the wild type *T. gondii* strains (Fox et al., 2009; Huynh and Carruthers, 2009). However, lack of Ku80 rendered these parasite strains more susceptible to double strand DNA breaks (Fox et al., 2009). Ku80 proteins have been implicated in a range of cellular activities such as telomere maintenance, tumor suppression, gene transcription regulation, heat shock induced response, and apoptosis (Fisher and Zakian, 2005). Because of these concerns, in some cases it may be useful to target wild type *T. gondii* strains, which still have Ku80. In other cases, researchers may need to perform transfection of strains for which Δ*ku80* is not available. In such wild type strains longer regions of homologous isogenic DNA are required for inducing homologous recombination.

Classical cloning techniques used for the engineering of tagging or gene knock out constructs require extensive sequence information of the target gene with compatible restriction sites. These requirements complicate the construction of the parent plasmid backbone when one wants to tag or delete more than one gene at a time.

To alleviate these “bottle-necks” we have devised an approach with the aid of Gateway® technology to efficiently, either delete or epitope tag a target gene at the chromosomal locus in a wild type *T. gondii* strain. This technology is a flexible and universal cloning approach based on lambda phage site specific recombination (Hartley et al., 2000). We used restriction enzyme mediated cloning with a multisite Gateway® system to tag *T. gondii* predicted gene 25.m01787 (coding for a homolog of yeast RNA polymerase II transcription factor Brf1) at the endogenous locus. The multisite Gateway® system with a PCR amplified construct can also be used to engineer deletion constructs with long regions of flanking sequences more efficiently. Finally, using *UPRT* as a test case, we demonstrate that gene deletion is simple and efficient by employing a split marker strategy.

2. Materials and methods

2.1. Parasite culture and purification

The *T. gondii* RH strain was maintained by serial passage at 37 °C 5% CO_2 in human foreskin fibroblasts (HFF) in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine and 5 mM penicillin/streptomycin (Invitrogen-GIBCO, Life Technology, Carlsbad, California). Infected cultures were lysed by passage through a 25-gauge needle, the released parasites purified by filtration through a 3 μm Nucleopore membrane, and then pelleted by centrifugation as published (Roos et al., 1994).

2.2. Generation of a plasmid template for the amplification of ORF25.m01787 3HA tagging cassette

The pTetO$_7$*SAG4SUB*2HA plasmid, in which Tg*SUB2* gene is fused at the C-terminus with a single influenza virus hemagglutinin (HA) epitope sequence at a *Nhe*1 restriction enzyme site was used as the parent plasmid for manipulation (Binder and Kim, 2004). The Gateway® cassette reading frame C.1 (RFC.1, Invitrogen, CA) was used as the template to amplify RFC.1 with oligonucleotides (RFC.1*Nsi*1For and RFC.1*Nhe*Rev) containing *Nsi*1 and *Nhe*1 restriction enzyme sites. The 1.7 Kb PCR product was ligated to the vector pTetO$_7$*SAG4SUB*2HA plasmid at *Nsi*1 and *Nhe*1 replacing *SUB*2HA fragment. The new plasmid pTetO$_7$*SAG4*RFC.1 was grown in *ccd*B survival bacterial cells (Invitrogen, CA) and selected in the presence of carbenicillin and chloramphenicol.

Two oligonucleotides encoding three tandem repeats of the HA epitope were designed with *Nhe*1 and *Pac*1 restriction sites and a stop codon in frame with the epitope encoding sequence before the *Pac*1 restriction site. Equimolar concentrations of the sense and antisense primers were mixed and then denatured at 94 °C and annealed at 25 °C. The double stranded oligonucleotide epitope module, allowing a C-terminal translational fusion of 3HA (YPYDVPDYA), was then inserted using a ligase reaction into the digested pTetO$_7$*SAG4*RFC.1 vector to generate pTetO$_7$*SAG4*RFC.13HA (Fig. 1A).

The plasmid template for genomic tagging of ORF 25.m01787 (renamed TgME49_007900 in Release 6 www.Toxodb.org) with the 3HA epitope was then engineered using three steps. In the first step a 1.5-kb genomic region of ORF25.m01787 gene upstream of the predicted stop signal was PCR amplified using forward and reverse primers (*Kpn*1*BRF*1For and *BRF*1*Nhe*Rev) with built in *Kpn*1 and *Nhe*1 sites as shown in Fig. 1B. After double digestion with the enzymes, the genomic DNA fragment was ligated into the pTetO$_7$*SAG4*RFC.13HA vector and then transformed into TOP10 chemically competent *E. coli* cells for selection in the presence of carbenicillin to yield a plasmid pORF3HA1. An 0.8-kb 3′ untranslated/flanking region (UTR) of ORF 25.m01787 immediately after the predicted stop codon was amplified with primers (*Pac*1*BRF*1For and *BRF*1*Sac*IIRev) containing *Pac*1 and *Sac*II restriction enzyme sites (Fig. 1B) using RH genomic DNA as the template. The purified PCR product was then ligated at the *Pac*1 and *Sac*II sites of pORF3HA1 to generate pORF3HA2.

In the second step, three entry vectors were generated using the Multisite Gateway Technology kit (Invitrogen, CA) by following the protocols supplied with the reagent. In brief, three PCR fragments: A, B and C each flanked by specific *att*B sites were generated (Fig. 1C). For the PCR fragment A, sense primer was a chimera of attB4 sequences (*att*B4*BRF*1For) followed by the genomic region of the ORF 25m.01787 just after the *Kpn*1 restriction site in the plasmid pORF3HA2. The antisense primer *att*B1*BRF*1Rev was designed to have attB1 sequences and a region of 3′UTR of the ORF25.m01787 just before the *Sac*II restriction site in the plasmid pORF3HA2. Using the above primer pair and pORF3HA2 as a template a 2.4 kb PCR product was then generated. The fragment B was a PCR product flanked by attB1 and attB2 sites and was amplified from a plasmid containing chloramphenicol acetyl transferase (CAT) gene cloned under the control of *T. gondii* tubulin promoter as a template using *att*B1*CAT*For and *att*B2*CAT*Rev primer pair (Soldati and Boothroyd, 1993). The DNA fragment C was a 1.5 kb genomic region downstream of 0.8 kb 3′UTR of the target ORF 25.m01787 (Fig. 1C) and was generated using *att*B2*BRF*1For and *att*B3*BRF*1Rev primers and thus is flanked by attB2 and attB3 sequences. Around 100 ng each of the purified PCR products were incubated with 150 ng of appropriate pDONR vectors respectively in the presence of 2 μl of BP clonase II enzyme mix. The BP clonase mediated recombination cloning yielded three kanamycin selectable Gateway entry vectors namely pENTR1, pENTR2 and pENTR3 containing fragments A, B and C respectively.

In step 3 of the generation of ORF 25.m01787 tagging construct, 20–25 fmol of the three entry vectors generated in the second step viz;