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RESEARCH ARTICLE

Efficient Editing of Malaria Parasite Genome Using the CRISPR/Cas9 System

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ABSTRACT Malaria parasites are unicellular organisms residing inside the red blood cells, and current methods for editing the parasite genes have been inefficient. The CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats and Cas9 endonuclease-mediated genome editing) system is a new powerful technique for genome editing and has been widely employed to study gene function in various organisms. However, whether this technique can be applied to modify the genomes of malaria parasites has not been determined. In this paper, we demonstrated that Cas9 is able to introduce site-specific DNA double-strand breaks in the *Plasmodium yoelii* genome that can be repaired through homologous recombination. By supplying engineered homologous repair templates, we generated targeted deletion, reporter knock-in, and nucleotide replacement in multiple parasite genes, achieving up to 100% efficiency in gene deletion and 22 to 45% efficiencies in knock-in and allelic replacement. Our results establish methodologies for introducing desired modifications in the *P. yoelii* genome with high efficiency and accuracy, which will greatly improve our ability to study gene function of malaria parasites.

IMPORTANCE Malaria, caused by infection of *Plasmodium* parasites, remains a world-wide public health burden. Although the genomes of many malaria parasites have been sequenced, we still do not know the functions of approximately half of the genes in the genomes. Studying gene function has become the focus of many studies; however, editing genes in malaria parasite genomes is still inefficient. Here we designed several efficient approaches, based on the CRISPR/Cas9 system, to introduce site-specific DNA double-strand breaks in the *Plasmodium yoelii* genome that can be repaired through homologous recombination. Using this system, we achieved high efficiencies in gene deletion, reporter tagging, and allelic replacement in multiple parasite genes. This technique for editing the malaria parasite genome will greatly facilitate our ability to elucidate gene function.

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Deletion or modification of a gene is a powerful approach to study gene function in many organisms. Various gene editing methods such as gene knockout (KO), gene tagging, or allelic replacement (AR) have been developed and widely used to study gene function in *Plasmodium* parasites (1–3). These methods are generally based on homologous recombination to insert a piece of DNA containing a drug selectable marker into a target gene or to replace a gene with a modified gene sequence. However, these approaches are time-consuming and inefficient, requiring a long period of drug selection and parasite cloning. Additionally, the site of DNA insertion or replacement is often not very specific, occurring at a random site of the homologous regions.

Recently, more efficient and site-specific genome editing technologies such as zinc finger nuclease (ZFN)-mediated modifications have been developed for studying genes in *Plasmodium falciparum* parasites (4, 5); however, limited choices of targeting sites and high cost have restricted the wide application of this technol-

ogy. Transcription activator-like effector nuclease (TALEN) (6, 7), another potent method that has been developed for genomic editing in many organisms, has not been successfully adapted for editing genes in *Plasmodium* parasites. More recently, another simple but powerful genome editing technology, CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats and Cas9 endonuclease-mediated genome editing), has been developed and successfully applied to modify the genomes of many organisms (8–12), including another apicomplexan parasite, *Toxoplasma gondii* (13). The CRISPR/Cas9 system was originated from a prokaryotic RNA programmable nuclease that can introduce a double-strand break (DSB) at a specific site on a chromosome through heterologous expression of two components: Cas9 nuclease and a targeting single guide RNA (sgRNA) (14). Target-specific DSBs introduced by the CRISPR/Cas9 system can be repaired either by activating the error-prone nonhomologous end-joining (NHEJ) pathway (15) or by homologous recombination if

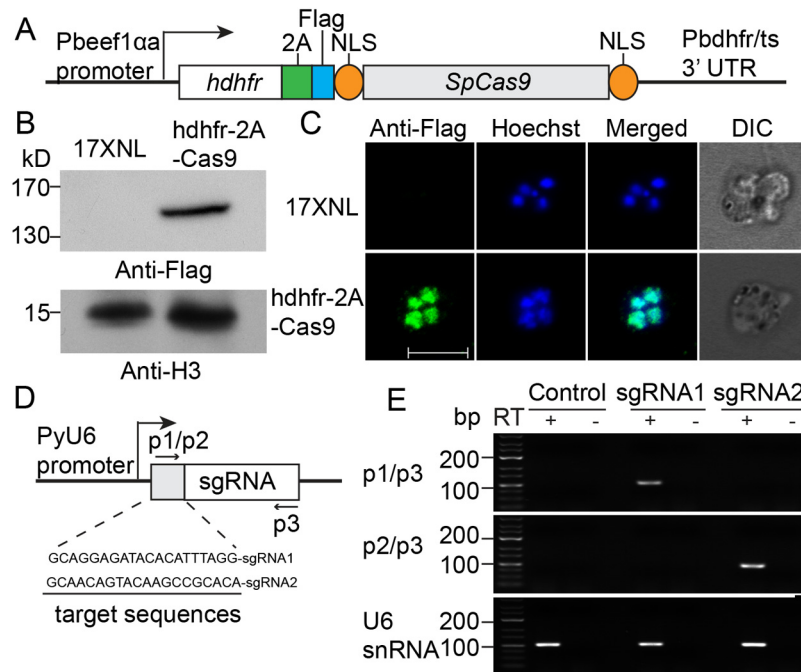


FIG 1 Coexpression of Cas9 and sgRNA in *Plasmodium yoelii*. (A) Schematic of a construct for coexpression of 2A-linked *hdhfr* and *SpCas9* genes driven by the *Pbef1α* promoter. (B) *SpCas9* expression detected using immunoblotting with anti-Flag antibody. *P. yoelii* 17XNL with no transfection was used as a control. Histone H3 was used as the loading control (anti-H3, anti-histone H3 antibody). The positions of molecular mass markers (in kilodaltons) are indicated to the left of the blots. (C) Nuclear localization of *SpCas9* detected using immunofluorescence with anti-Flag antibody. Nuclei were stained with Hoechst 33342. 17XNL is the untransfected parasite control. DIC, differential interference contrast. Bar = 5 μm. (D) Schematic of sgRNA construct driven by the *P. yoelii* U6 snRNA promoter. The protospacer sequences (20 nucleotides [nt]) of sgRNA1 and sgRNA2 are indicated. p1/p3 and p2/p3 are the forward and reverse primer pairs for detecting sgRNA1 and sgRNA2 transcript, respectively. (E) sgRNA transcripts detected using RT-PCR. PCR amplification of the cDNA after reverse transcription (+) and PCR amplification of the RNA without reverse transcription (–) are indicated. Endogenous U6 snRNA serves as an internal control. The positions of molecular size markers (in base pairs) are indicated to the left of the blots.

a donor template is provided (16). The CRISPR/Cas9 system has been shown to be more efficient than the other techniques in generating gene knock-in (KI), KO, or AR in many organisms and has revolutionized many fields of biomedical research (17). It is therefore desirable to develop a CRISPR/Cas9-based genome editing method for studying gene function of malaria parasites. However, malaria parasites reside within red blood cells (RBCs), and a piece of foreign DNA has to pass four layers of membranes (RBC membrane, parasitophorous membrane, parasite cytoplasm membrane, and the parasite nuclear membrane) to reach the parasite nuclei. The efficiency of transfection of parasites has been low compared with the efficiency of transfection of mammalian cells. Additionally, because of its parasitic lifestyle, a malaria parasite has a “reduced” genome encoding ~6,500 predicted genes. Many enzymes in eukaryotic cells are often absent in the parasites. For example, the enzymes required for RNA interference (RNAi) have not been found, and the use of RNAi to silent gene expression in malaria parasites has not been possible so far (18). Whether the CRISPR/Cas9 system can be employed to edit the genomes of malaria parasites remains an open question, and intense efforts are ongoing in the malaria community to answer this question.

In this study, we report the development of a method based on the CRISPR/Cas9 system to efficiently edit the genome of *Plasmodium yoelii*, including KO, KI, or AR multiple *P. yoelii* genes with high efficiency and accuracy. Using this powerful technique to edit the malaria parasite genome will greatly facilitate our ability to

elucidate gene function, and hopefully to control this deadly disease.

RESULTS

Construction of a CRISPR/Cas9 system for modification of the *P. yoelii* genome. CRISPR/Cas9-mediated genome editing requires expression of two components: Cas9 nuclease and a targeting single guide RNA (sgRNA), which form a complex to induce a double-strand break (DSB) at the targeted site. To reduce the size of the plasmid construct and to overcome the problem of limited selectable markers available for *P. yoelii*, we constructed an expression plasmid containing the human dihydrofolate reductase (*hdhfr*)-2A peptide-gfp genes under the *Plasmodium berghei eef1α* (*Pbef1α*) promoter and showed bicistronic expression of both genes after introduction into the *P. yoelii* 17XNL strain (see Fig. S1 in the supplemental material). The viral “ribosome skip” 2A peptide has been shown to coordinate coexpression of two individual genes under a single promoter in *P. falciparum* (4). We next generated transgenic parasites transiently expressing *hdhfr*-2A-Flag-S. *pyogenes* Cas9 (*SpCas9*) fusion driven by the *Pbef1α* promoter (Fig. 1A). Immunoblot detection of the Flag tag fused to the Cas9 gene downstream of the 2A peptide revealed an expected 163-kDa product, suggesting expression of Cas9 (Fig. 1B). Because Cas9 is a nuclease functioning within the nucleus, we attached two nuclear localization signals (NLSs) to the 5' and 3' of the Cas9 gene to direct the protein to the nucleus. As expected, expression of Cas9 was detected within the parasite nucleus using

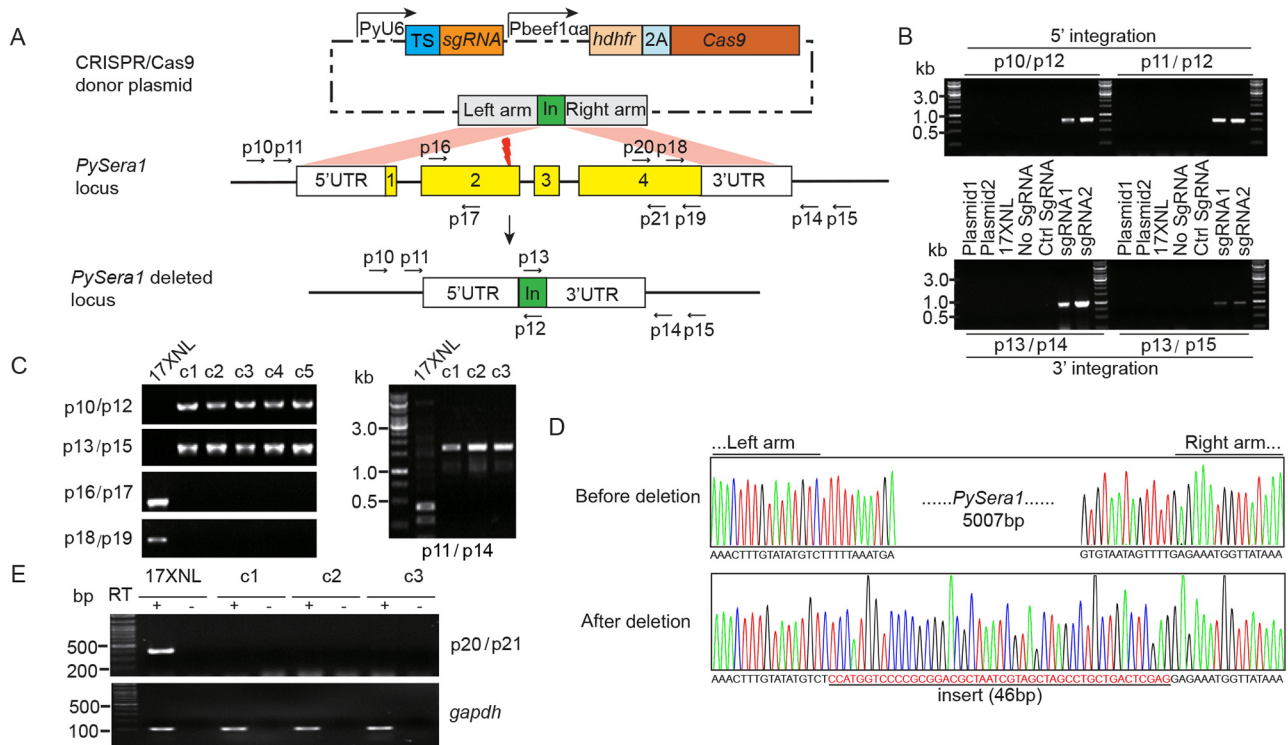


FIG 2 CRISPR/Cas9-mediated deletion of *Plasmodium yoelii* *sera1* gene. (A) Schematic construct for disrupting the *PySera1* gene. The plasmid contains Cas9 and sgRNA expression cassettes and donor template for HR repair after a double-strand break (DSB) at the 3' end of the *PySera1* exon 2 (red thunderbolt). The DNA inserted (In) between the left and right arms was added to detect donor integration in the design of the PCR primers. Exons 1 to 4 are indicated by the yellow boxes. TS (blue box) indicates the sgRNA target sequence. The positions and directions of primers p10 to p21 are indicated by the small black arrows. (B) PCR analysis of 5' and 3' integrations in *P. yoelii* 17XNL parasite and plasmid-transfected uncloned cultures. The positions of primers (e.g., p10/p12) are shown in panel A. Ctrl, control. (C) PCR screening of clonal parasites for targeted *PySera1* deletion. DNAs from five individual clones (c1 to c5) and from 17XNL parasite were screened. (D) DNA sequencing confirms a 5.0-kb deletion in the *PySera1* gene from clonal parasite c1. The top panel shows the partial nucleotide sequence of the left and right arms from parental strain 17XNL. The bottom panel shows the 46-bp DNA insert between the left and right arm sequences in clone c1. (E) RT-PCR of *PySera1* mRNA from parental strain 17XNL and clones c1, c2, and c3, showing the lack of *PySera1* transcription in the three cloned lines. PCR amplification of the cDNA after reverse transcription (+) and PCR amplification of the RNA without reverse transcription (–) are indicated. *P. yoelii* *gapdh* mRNA serves as the endogenous control.

anti-Flag antibody in an immunofluorescence assay (IFA) (Fig. 1C). In mammalian systems, sgRNA is synthesized by RNA polymerase III, and transcription is driven by a U6 small nuclear RNA (snRNA) promoter (8, 9). By searching the *P. yoelii* genome database, we identified a U6 snRNA homolog and cloned a 350-base-pair (bp) segment upstream of the transcriptional start site of U6 snRNA to function as a promoter (Fig. S2). To test the capability of the *P. yoelii* U6 (*PyU6*) promoter to drive the transcription of sgRNA, we transfected parasites with plasmids containing either sgRNA1 or sgRNA2, which differ by 20-bp protospacer sequences, and detected the transcript of the 98-bp sgRNA driven by the *PyU6* promoter (Fig. 1D and E), confirming the activity of the *PyU6* promoter in driving sgRNA expression. Because DNA DSBs are repaired by homologous recombination (HR) pathways in *Plasmodium* (4, 19), donor templates were provided for HR repair for all the modifications in this study. We next constructed a Cas9-sgRNA plasmid containing both the *hdhfr*-2A-*SpCas9* and *PyU6*-sgRNA cassettes with cloning sites for the insertion of donor template sequences (Fig. 2A, 3A, and 4A). This vector was named pYC (for plasmid for *P. yoelii* CRISPR/Cas9).

Cas9-mediated gene deletion after homologous repair. We next attempted to delete the parasite gene encoding a serine protease (*P. yoelii* *sera1* [*PySera1*]) that has been shown to be non-

essential for the growth of blood stages (20). We constructed a plasmid pYC-*PySera1* containing a 46-bp tag DNA (for PCR primers) flanked by two homologous regions of *PySera1* (0.7 kb of the 5'-flanking region and 0.8 kb of the 3'-flanking region) (Fig. 2A). Considering potential variation in target site accessibility by the Cas9/sgrRNA complex, we designed two sgRNAs to target the 3' end of the *PySera1* exon 2, generating plasmids pYC-*sera1*-sgRNA1 and pYC-*sera1*-sgRNA2. One day after electroporation of the plasmids into the *P. yoelii* 17XNL strain, parasites were selected with pyrimethamine (Pyr) supplied in drinking water. Pyr-resistant parasites were observed microscopically 5 to 7 days after electroporation. PCR analysis of genomic DNA from parental strain 17XNL and plasmid-transfected parasites indicated successful integration of left and right homologous arms at specific sites directed by sgRNA1 and sgRNA2, respectively (Fig. 2B). Two independent transfections with pYC-*sera1* plasmids without sgRNA or with random sgRNA failed to yield parasites with *PySera1* deletion even after 10 days (Fig. 2B), indicating events specifically mediated by the Cas9/sgrRNA complex. After limiting dilution cloning and injection of 21 mice, we obtained 5 clones, all of which (100%) had targeted integration of donor template and expected genomic deletion by PCR analysis (Fig. 2C). DNA samples from three clones (Δ *sera1*-c1/c2/c3) were sequenced to con-

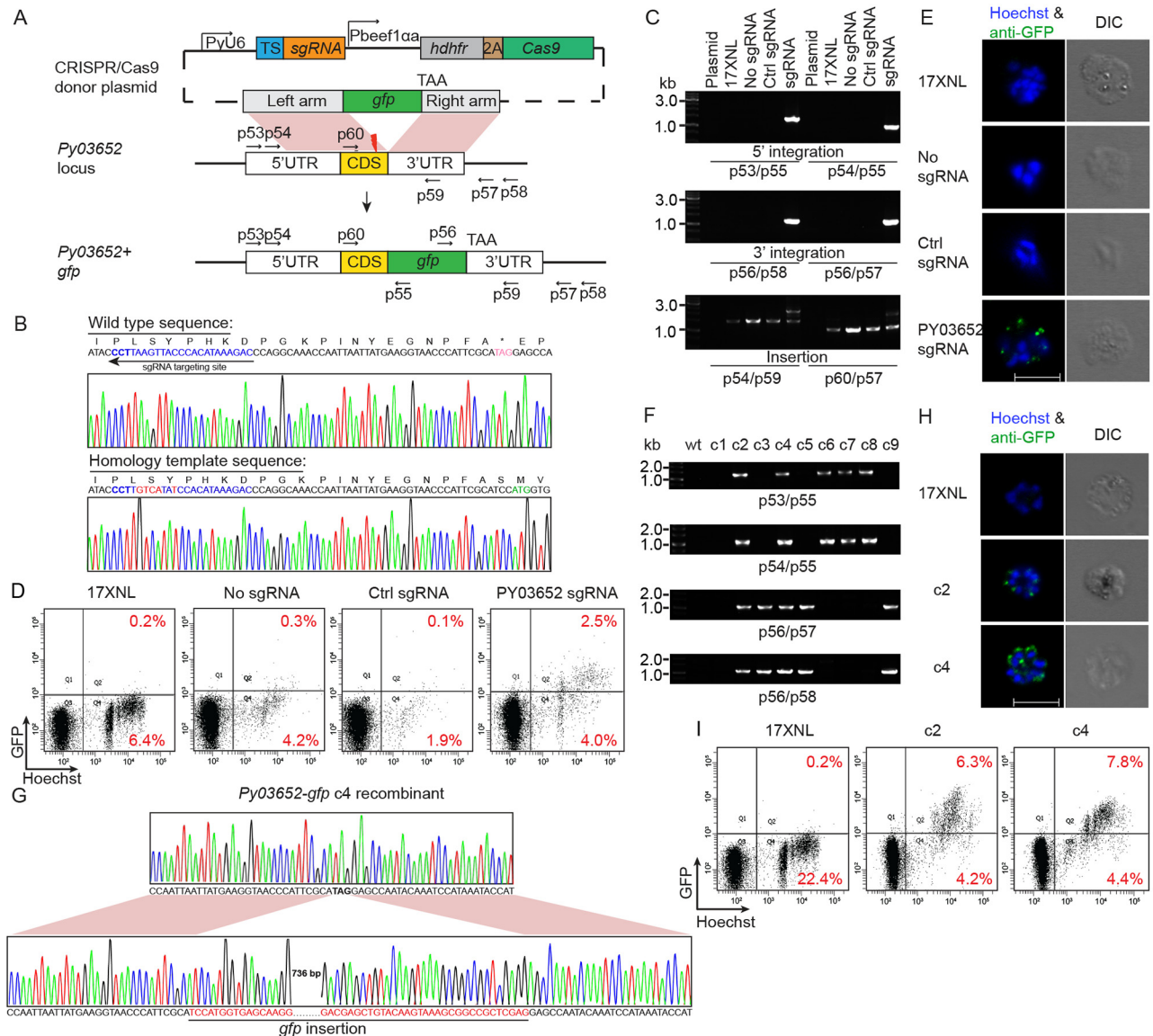


FIG 3 Tagging of the endogenous *Py03652* gene with the *gfp* gene in *Plasmodium yoelii*. (A) Schematic construct for tagging *Py03652* with *gfp*. The plasmid contains the Cas9 and sgRNA expression cassettes and donor template for HR repair after double-strand break (DSB) targeting the *Py03652* C-terminal part of the coding sequence (CDS) (red thunderbolt). The directions and positions of primers p53 to p60 are indicated by the small black arrows. (B) The homologous left arm in the donor template sequence is identical to the genomic sequence but contains five silent nucleotide substitutions (red) at the sgRNA-binding site (blue) to prevent binding and cleavage of the integrated DNA by the Cas9/sgRNA complex. The sequence of the protospacer adjacent motif (PAM) is in bold type. The sequence of the protospacer adjacent motif (PAM) is in bold type. The sequence of the protospacer adjacent motif (PAM) is in bold type. (C) PCR detection of 5' and 3' integration of *gfp* into the *P. yoelii* 17XNL genome from uncloned parasites. (D) FACS detection of parasites expressing GFP-tagged PY03652 protein. No GFP expression in the control groups (group with no sgRNA or with random sgRNA). (E) Immunofluorescence of parasites with or without PY03652-GFP integration, as detected using anti-GFP antibody. Nuclei were stained with Hoechst 33342 (blue). Bar = 5 μ m. (F) PCR detection of clonal parasites with targeted *gfp* tagging. wt, wild type. (G) DNA sequencing confirmation of *gfp*-tagged *Py03652* gene in clonal parasite c4. The top panel shows the nucleotide sequence between the left and right arms from parental strain 17XNL, and the bottom panel shows the *gfp* tag (shown in red) insertion at the C-terminal region of *Py03652* in clone c4. The stop codon is shown in bold type. (H) Immunofluorescence detection of PY03652-GFP in clones c2 and c4 using anti-GFP antibody. 17XNL is the negative control. Bar = 5 μ m. (I) FACS analysis confirming PY03652-GFP expression in parasite clones c2 and c4.

firm the targeted deletion of a 5.0-kb coding region of *Pysera1* (Fig. 2D). *Pysera1* expression in the modified parasite clones was undetectable at the mRNA level using reverse transcriptase PCR (RT-PCR) analysis (Fig. 2E).

To further confirm the general usage of the CRISPR/Cas9 system for gene deletion, we applied this method to delete another two genes: *Pysera2*, which encodes another *Plasmodium* serine

protease expressed in the asexual blood stages, and *PyPDH/E1 α* , which is required for liver stage development (20, 21). Deletion mutations of both *Pysera2* and *PyPDH/E1 α* genes were detected in the CRISPR/sgRNA-transfected parasites (see Fig. S3 in the supplemental material). Targeted deletions of *Pysera2* coding region (3.9 kb) and *PyPDH/E1 α* coding region (1.6 kb) were both confirmed using DNA sequencing from transfected parasite mixtures

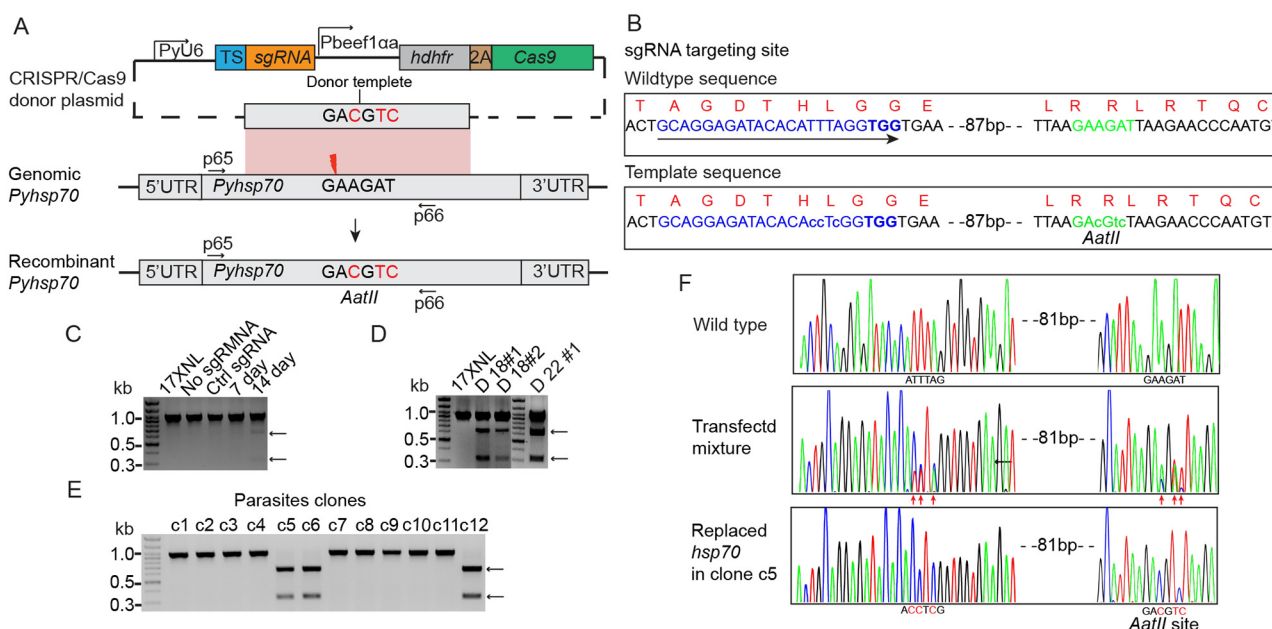


FIG 4 Targeted nucleotide replacement in *Plasmodium yoelii* heat shock protein 70 gene (*Pyhsp70*). (A) Schematic construct for *Pyhsp70* nucleotide replacement. The homologous donor template comprises a fragment of *Pyhsp70* spanning 340 bp upstream and 620 bp downstream of the Cas9 target site (red thunderbolt). (B) The donor template sequence is identical to the genomic sequence but contains three nucleotide substitutions (green lowercase letters) that create an AatII restriction site for detecting modification by restriction enzyme digestion. In addition, three nucleotides at the sgRNA-binding site in the donor sequence are mutated (blue lowercase letters). The sequence of the protospacer adjacent motif (PAM) is shown in bold type. (C) Gene replacement was detected in uncloned parasites 14 days after electroporation. No recombinant was detected with the parasite transfected with plasmid without sgRNA or with random sgRNA. The small black arrows indicate the DNA bands (620 bp and 310 bp) digested with AatII from the PCR product of the recombinant genome. (D) Increased intensities of digested bands (arrows) from parasites 18 (#1 and #2) were parasites from two infected mice and 22 days postinfection. (E) RFLP screening of clonal parasites with targeted gene replacement (two bands). (F) DNA sequencing confirmation of allelic replacement. The top panel shows the nucleotide sequence from strain 17XNL; the middle panel shows the double peak (red arrow) of targeted nucleotide from transfected parasite mixtures; the bottom panel shows targeted mutations at both AatII and sgRNA-binding site in clone c5.

and seven *Pysera2*-disrupted clones after limiting dilution cloning (Fig. S3D and S3H). These results demonstrated that desired deletion of *P. yoelii* genes at specific sites could be successfully achieved via homologous repair of DSBs introduced by CRISPR/sgRNA.

Cas9-mediated reporter “knock-in.” Tagging endogenous genes with fluorescent proteins or reporters is often used for studies of protein subcellular localization and protein interaction. To test whether Cas9-mediated HR could be used to insert tags into endogenous genes, we built a construct (pYC-*Py03652-gfp*) containing a 710-bp C-terminal region of the *Py03652* gene followed by the *gfp* gene and a 779-bp 3′ untranslated region (3′ UTR) of the *Py03652* gene (Fig. 3A). The *Py03652* gene encodes an early transcribed membrane protein with unknown function (ETRAMP) (22). To prevent binding and cleavage of the integrated DNA by the Cas9/sgRNA complex, we introduced five silent nucleotide substitutions at the sgRNA target site in the donor template (Fig. 3B). We obtained parasites with integration of donor template into the 3′ end of *Py03652* 5 days after transfection (Fig. 3C). In contrast, transfections with pYC control plasmid or pYC-*Py03652-gfp* without sgRNA or with random sgRNA failed to yield parasites with targeted recombination 10 days after electroporation (Fig. 3C). The expression of recombinant PY03652-green fluorescent protein (GFP) protein was detected in ~38% of the infected RBCs using fluorescence-activated cell sorting (FACS) analysis and IFA (Fig. 3D and E). After limited dilution cloning, we obtained nine parasite clones from 22 parasite-

injected mice. Two clones (*Py03652-gfp*-c2 and -c4) out of the nine clones (22%) had the expected replacement of the endogenous locus by the donor *Py03652-gfp*, as detected using site-specific PCR and DNA sequencing (Fig. 3F and G), and the expression of GFP was further confirmed using FACS analysis (Fig. 3I). In asexual blood stages, PY03652-GFP is localized to the parasite periphery as punctate dots (Fig. 3E and H), which is consistent with the protein localization reported previously (22).

For HR-based genome modification, long donor template is often used to increase the targeting efficiency (23); however, for some genes, long donor sequences may not be obtained easily because of difficulty in cloning AT-rich DNA from many *Plasmodium* genomes. We next investigated whether the length of template DNA could influence KI efficiency using two pairs of templates with different lengths (5′ 345 bp and 3′ 412 bp in Fig. S4 in the supplemental material and 5′ 710 bp and 3′ 779 bp in Fig. 3). We obtained integration of 28.2% and 45.4% for the short and long templates ($P < 0.001$ by *t* test), respectively (Fig. S4D). Comparable results were obtained in another set of experiments introducing myc-tagged PY03652 using both the long (710- and 779-bp) or short (345- and 412-bp) donor templates (Fig. S5). These results demonstrate that the CRISPR/Cas9 system can be used to efficiently tag genes of interest in *P. yoelii*, and longer templates should be used if possible.

Cas9-mediated nucleotide replacement. Targeted nucleotide substitution is widely used to characterize the functional roles of specific amino acids in a gene. To test whether a precise nucleotide

TABLE 1 Numbers of mutations detected at the potential off-target cleavage sites in the *Plasmodium yoelii* genome

sgRNA	Sequence ^a	No. of mismatches	Chromosome location ^b	Mutation
<i>Pysera1</i> targeting sgRNA1	GTACCGGGAACTCTGATCAAGG	None		
	ACAAAA <u>GCCTT</u> CTGATCAAGG	9	13: 2055967–2056749(–)	None detected
	GATATT <u>GAAAA</u> ATCTGATCATGG	7	10: 899397–901846(–)	None detected
	ATTTTAAAA ATCTGATCATGG	8	10: 1255700–1258432(–)	None detected
<i>Py03652</i> targeting sgRNA	GTCTTTATGTGGGTAACCTTAAGG	None		
	TTAAATAA <u>TATT</u> GTAACCTTATGG	9	04: 309670–311468(+)	None detected
	TTTCTATT <u>TCTG</u> TAACCTTATGG	7	11: 790345–792444(+)	None detected
	TTAAATTT <u>TTTC</u> GTAACCTTATGG	9	04: 529947–530873(–)	None detected
	GT TTTTTC <u>ACTA</u> GTAACCTT G GGG	8	04: 995696–1000093(–)	None detected
	ATGTTTTTT <u>TTTC</u> AGTAACCT CC GGG	8	11: 1701710–1706203(–)	None detected

^a Nucleotides in bold type are mismatches between the sgRNA sequences and the potential off-target sites. Underlined nucleotides are nucleotides in the protospacer adjacent motif (PAM) following the 20-nt sgRNA targeting sequence.

^b The chromosome number is shown before the colon. The numbers after the colon are the positions on the chromosome. Minus and plus symbols in parentheses indicate the forward or reverse strand of genome DNA, respectively.

substitution could be introduced at a desired site of the genome, we designed a construct to introduce silent mutation of nucleotides into the coding region of the *Pyhsp70* gene. We first generated a donor template containing three silent nucleotide substitutions, creating an AatII site into the *Pyhsp70* coding region for easy restriction fragment length polymorphism (RFLP) analysis (Fig. 4A). One sgRNA was designed to target the site 100 bp upstream of the AatII site (Fig. 4B). To avoid donor DNA digestion by Cas9/sgRNA effector, three nucleotide substitutions were introduced at the binding site of sgRNA in the donor template (Fig. 4B). Pyr-resistant parasites emerged 5 to 7 days after electroporation, and by RFLP gel analysis, we clearly detected replacement events specifically in the transfected parasites 14 days after electroporation (Fig. 4C). Independent transfections with pYC plasmids without sgRNA or with random sgRNA failed to yield detectable replacement at day 14 (Fig. 4C). The parasite populations with introduced restriction site increased 18 and 22 days posttransfection (Fig. 4D). In a clonal analysis, we detected replacement events in parasites from 3 of 12 (25%) positive mice after injecting 32 mice (Fig. 4E). All three clones were confirmed to have correct replacement of targeting sequence after DNA sequencing (Fig. 4F). These results show that site-specific nucleotide substitutions can be generated efficiently using this method.

Assessment of Cas9 specificity in *P. yoelii*. One concern using CRISPR/Cas9-mediated genome editing is the induction of DSBs at off-target sites. Previous studies of other organisms indicated that Cas9 could introduce a DSB at sequences with as many as five mismatches of the target sequence (24, 25). To evaluate the site specificity, we searched the 17XNL genome for potential sequences homologous to the target site and include a protospacer adjacent motif (PAM) sequence in experiments of *Pysera1* deletion and *Py03652* tagging with *gfp*. We did not identify any genomic off-target sequences sharing fewer than six nucleotide mismatches. When search criteria were relaxed (6 to 10 mismatches), we found three and five potential off-targets in *Pysera1* and *Py03652* sgRNA targeting sites, respectively (Table 1). We PCR amplified the potential off-target regions from genomic DNA from two *Pysera1* deletion clones (c1 and c2) and two *Py03652-gfp* tagging clones (c2 and c4). DNA sequencing of the PCR products detected no mutations at any of these sites (Table 1; see Fig. S6 in the supplemental material), suggesting no off-target mutation by CRISPR/Cas9-mediated modification. The relative

smaller genome size and lack of NHEJ DNA repair pathway in malaria parasites may reduce the chance of off-target events (19).

DISCUSSION

In this study, we developed a CRISPR/Cas9-based genome editing system and successfully applied the system to modify the rodent malaria *P. yoelii* genome, which is an important model commonly used for studying malaria biology and malaria parasite and host interaction. We showed that the system could be used to KO, KI, or replace nucleotides in the parasite genes with high efficiency and specificity (100% for each *Pysera1* and *Pysera2* gene deletion, 22 to 45% in tagging *Py03652* with *gfp* and 25% in nucleotide replacement of the *hsp70* locus) (see Table S1 in the supplemental material). The development of this system will greatly improve our ability to modify the parasite genome and impact the functional studies of malaria parasites. Our results definitely proved that the CRISPR/Cas9 system is functional in *P. yoelii*. It would be interesting to apply this technique to other malaria parasites such as the human malaria parasite *P. falciparum*.

The pYC plasmid we developed contains Cas9/sgRNA cassettes with targeting sgRNA protospacer and restriction sites for cloning DNA templates, allowing construction of a single vector for various gene modification strategies. In addition to high efficiency and site specificity, the Cas9/sgRNA method has other advantages compared with the traditional gene editing methods. First, the conventional method of gene modification based on HR generally requires insertion of selection markers and other plasmid elements into the targeting locus of the parasite genome, which may influence the expression of other genes near the targeted locus. Gene editing by homologous repair of DSBs introduced by CRISPR/Cas9 requires only transient expression of Cas9/sgRNA from a plasmid; no integration of selection marker or other plasmid elements into the target site occurs, reducing the unexpected impacts of foreign DNA on the parasite genome. Second, targeted modification of multiple genes is increasingly preferred in various functional studies such as deletion of multiple copies of genes in a gene family. The insertion of drug selection markers in the target site will restrict the number of genes that can be modified, because limited drug markers are available for malaria parasites. The CRISPR/Cas9 system we developed will allow modification of multiple genes without worrying about running out of drug selection markers. Third, we also demonstrated that short (300- to

400-bp) homologous templates were efficient in mediating recombination repair. Using the traditional method, longer homologous sequences are often preferred because of low recombination and transformation frequencies (23). This high efficiency of gene modification using short template sequences will benefit studies of AT-rich genomes such as *P. falciparum* or *P. yoelii* genome, because long AT-rich DNAs are often unstable in *Escherichia coli* and are difficult to clone into plasmid vectors.

In this study, we achieved high efficiency (100%) in deleting three gene segments with sizes ranging from 1.6 to 5.0 kb. However, lower efficiencies were observed in gene tagging (22 to 45%) and nucleotide replacement (~25%). Although we do not know the exact reasons for the lower efficiencies in gene tagging and nucleotide replacement, the introduction of nonsense nucleotide substitutions to alter the sgRNA targeting sites and/or to create restriction sites in the homologous sequences in these two types of experiments could contribute to the lower efficiencies observed. In the three gene deletion experiments, the DNA sequences of both left and right homologous arms were identical to the corresponding genomic DNA sequences. In the *Py03652* tagging experiment, we introduced five nonsense nucleotide substitutions at the sgRNA-binding site in the left arm of the donor template. In the *Pyhsp70* nucleotide replacement, we replaced three nucleotides at the sgRNA-binding site and another three nucleotides to create an AatII site in the homologous template. These substitutions might affect the stability of DNA pairing and/or the efficiency of homologous recombination, leading to lower efficiency in obtaining clones with the targeted modifications. Another possible explanation for lower efficiency in gene modification could be due to functional constraint to some specific genes, i.e., some genes contribute more than others to parasite survival and are more difficult to modify. Additional experiments are necessary to reveal the exact reasons for the lower efficiencies in our gene tagging and nucleotide replacement experiments. Nonetheless, the efficiencies of our gene tagging and nucleotide replacement are still higher than those obtained using the “traditional” homologous recombination and drug selection.

MATERIALS AND METHODS

Plasmid construction. To test whether *Thomomys asiana* virus 2A peptide can drive expression of two genes at the same time in *Plasmodium yoelii*, we generated a *Pbeef1aa* promoter-*hdhfr-2A-gfp-Pbdhfr/ts* 3' UTR plasmid (see Fig. S1A in the supplemental material) derived from plasmid pL0019 obtained from the Malaria Research and Reference Reagent Resource Center (MR4) (<http://www.mr4.org>) (8). *SpCas9* nuclease coding fragment was amplified from plasmid 42230 obtained from Addgene, containing N-terminal triple Flag tag and simian virus 40 (SV40) nuclear localization signals (NLS) on both sides. Replacement of *gfp* in the *Pbeef1aa* promoter-*hdhfr-2A-gfp-Pbdhfr/ts* 3' UTR plasmid with *Cas9* generated plasmid *Pbeef1aa* promoter-*hdhfr-2A-Cas9-Pbdhfr/ts* 3' UTR cassette using ClaI and AgeI restriction sites. Potential U6 snRNA promoter was PCR amplified from genomic DNA from *P. yoelii* 17XNL strain using the primers PyU6proF/PyU6proR (see Table S2 in the supplemental material). DNA fragment encoding sgRNA scaffold was also amplified from plasmid 42230 (8) and ligated with U6 snRNA promoter to generate the U6 promoter-sgRNA cassette (Fig. 1D). An annealed oligonucleotide pair encoding sgRNA targeting sequence was inserted in the dual BsmBI sites upstream of the sgRNA scaffold, leading to a plasmid containing both the *hdhfr-2A-SpCas9* and *PyU6-sgRNA* cassettes, as well as the sites for HR donor template insertion. This plasmid was named pYC (for plasmid for *P. yoelii* CRISPR/Cas9).

To generate the Cas9-sgRNA vector for gene deletion, including dele-

tion of *Pysera1* (Gene ID in PlasmoDB database PY17X_0305700), *Pysera2* (PY17X_0305600), and *PDH/E1 α* (PY17X_0925800), we first amplified the 5' - and 3' -flanking genomic regions (500 to 800 bp) as left and right homologous arms by PCR using the primers listed in Table S2 in the supplemental material. The left and right arms were inserted into the linker site in the pYC plasmid. To detect HR, a 46-bp DNA fragment was inserted between the left and right arms as a template for designing primers p12 and p13 (Table S2).

To generate the Cas9-sgRNA vector for tagging gene *Py03652* (PY17X_0946500) with *gfp* or *myc* tags, we first amplified the C-terminal part (300 to 800 bp) of the coding region as the left arm and 400 to 800 bp from the 3' UTR region following the translation stop codon as the right arm using the primers listed in Table S2 in the supplemental material. A DNA fragment encoding the *gfp* or *myc* tag was inserted between the left and right arms in frame with the gene of interest. For each gene, one sgRNA was designed to target the site close to the C-terminal part of the coding region. To avoid binding of sgRNA to the target site in the left arm of the donor template after integration, three nucleotides in the sgRNA-binding site were replaced.

To generate the Cas9-sgRNA vector for targeted nucleotide replacement in the *Pyhsp70* (PY17X_0712100), we first PCR amplified approximately 1 kb of the coding region (with the Cas9-sgRNA cleavage site in the center of the region) as the donor template using primers listed in Table S2 in the supplemental material. Two or three nucleotide substitutions to create restriction sites without change of amino acids were introduced using synthetic oligonucleotides and PCR amplification. For each gene, one sgRNA was designed to target the site close to the restriction enzyme cutting site (Table S3). To prevent binding of sgRNA to the target site in the donor template, 3 nucleotide mutations were also introduced into the sgRNA-binding site in the template. All plasmids used in this study are available from the authors upon request.

Parasites used and parasite transfection. All transgenic *P. yoelii* parasites were generated from *P. yoelii* 17XNL strain. The parasites were propagated in Kunming (KM) outbred mice purchased from the Animal Care Center of Xiamen University. All mouse experiments were performed in accordance with approved protocols (XMULAC20140004) by the Committee for Care and Use of Laboratory Animals at the School of Life Sciences of Xiamen University. The procedures for parasite transfection, selection, and cloning were described previously (26) and illustrated in Fig. S7 in the supplemental material. Briefly, parasites were electroporated with purified circular plasmid DNA. Transfected parasites were intravenously injected into new mice, and parasites were selected with pyrimethamine (Pyr) in drinking water 1 day after injection. Parasites with transfected plasmids usually appear 5 to 7 days after injection. In allelic replacement experiments, parasite-infected RBCs were transferred to new mice when the parasitemia declined to ~0.5%.

DNA analysis. Blood samples from infected mice were collected from the orbital sinus, and RBCs were lysed using 1% saponin in phosphate-buffered saline (PBS). Parasite genomic DNAs were isolated using DNeasy blood kits (Qiagen) after washing off the hemoglobin, and these genomic DNAs were used in PCR amplifications. For gene deletion or gene tagging, targeted modification was confirmed by PCRs using two or three pairs of primer to detect 5' and 3' integrations. For targeted nucleotide substitutions, PCR-RFLP was used to detect introduced restriction sites in the transfected parasite mixtures. PCR products were purified and digested with restriction enzymes (AatII used for *Pyhsp70* replacement). Some PCR products were also sequenced directly without cloning into a plasmid vector.

RNA preparation and RT-PCR. Parasite pellets after lysis of RBCs were mixed with TRIzol (Invitrogen), and RNA was extracted according to the manufacturer's instructions (Qiagen). RNA quantitation was done using Nanodrop (Thermo). Purified RNA was treated with DNase using Turbo DNA-free kit (Life Technologies). cDNA was synthesized using RevertAid reverse transcriptase (Fermentas). Unique primers were designed for reverse transcription or for amplifying short regions of target genes.

Western blotting. Total proteins extracted from parasite pellets were separated on 4 to 15% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The blot was incubated with blocking buffer (PBS with 3% bovine serum albumin [BSA]) at room temperature for 1 h and then incubated at 4°C overnight with anti-Flag (mouse; 1:5,000; Abmart), anti-GFP (rabbit; 1:1,000; Cell Signaling), anti-histone H3 (rabbit; 1:1,000; Cell Signaling), or anti-Myc (rabbit; 1:1,000; Cell Signaling). Antibody to histone H3 was used as a control. Horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies (Sigma) were incubated with PVDF membranes for 2 h at room temperature before three washes with blocking and enhanced chemiluminescence (ECL) detection.

Fluorescence analysis of recombinant parasites. For live-cell imaging, parasite-infected mouse blood was washed twice with PBS before staining the cells with 2 µg/ml Hoechst 33342 (Sigma) (in PBS). The cells were applied to poly-L-lysine-coated glass-bottom culture dishes (NEST, China).

For fixed-cell imaging, the parasites were washed twice with PBS and were fixed with 4% paraformaldehyde–0.0075% glutaraldehyde on poly-L-lysine-coated glass slides for 30 min. After 3 washes in PBS, the parasites were treated with 0.1% Triton X-100 in PBS, blocked with 3% BSA in PBS, and stained with the primary antibody (mouse anti-Flag [1:1,000; Abmart], rabbit anti-GFP [1:500; Cell Signal], rabbit anti-Myc [1:500; Cell Signal]) at 4°C overnight. After 3 washes with blocking buffer, the samples were incubated with goat anti-mouse or anti-rabbit antibody labeled with Alexa Fluor 488 (1:2,000; Invitrogen) at room temperature for 1 h. Cells were stained with Hoechst 33342 to visualize nuclei. All images were captured and processed using identical settings in the Zeiss LSM 780 laser scanning confocal microscope with a 100×/1.49-numerical-aperture (NA) oil objective. Duplicate cultures were examined, and similar results were obtained in at least three independent experiments. To quantify the proportion of GFP-positive parasites after *Py03652-gfp* tagging, parasites were washed with PBS and analyzed with a flow cytometer. Cell nuclei were first stained with 2 µg/ml Hoechst 33342 (Sigma) in PBS. After a single wash, 20,000 cells were counted on a BD LSR Fortessa flow cytometer. Data were analyzed with FlowJo 7.6.3 with gating for nuclear stain Hoechst 33342 (FL1) and for green fluorescence (FL4).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01414-14/-/DCSupplemental>.

- Figure S1, PDF file, 1 MB.
- Figure S2, PDF file, 0.4 MB.
- Figure S3, PDF file, 0.6 MB.
- Figure S4, PDF file, 1.5 MB.
- Figure S5, PDF file, 1.1 MB.
- Figure S6, PDF file, 0.3 MB.
- Figure S7, PDF file, 0.1 MB.
- Table S1, PDF file, 0.1 MB.
- Table S2, PDF file, 0.1 MB.
- Table S3, PDF file, 0.2 MB.

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