International Journal for Parasitology 49 (2019) 705–714

Contents lists available at ScienceDirect

International Journal for Parasitology

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

Generation and functional characterisation of *Plasmodium yoelii csp* deletion mutants using a microhomology-based CRISPR/Cas9 method

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ARTICLE INFO

Article history:
Received 23 January 2019
Received in revised form 1 April 2019
Accepted 5 April 2019
Available online 13 June 2019

Keywords:
Rodent malaria
MMEJ
Double strand break
Circumsporozoite surface protein
Mosquito

ABSTRACT

CRISPR/Cas9 is a powerful genome editing method that has greatly facilitated functional studies in many eukaryotic organisms including malaria parasites. Due to the lack of genes encoding enzymes necessary for the non-homologous end joining DNA repair pathway, genetic manipulation of malaria parasite genomes is generally accomplished through homologous recombination requiring the presence of DNA templates. Recently, an alternative double-strand break repair pathway, microhomology-mediated end joining, was found in the *Plasmodium falciparum* parasite. Taking advantage of the MMEJ pathway, we developed a MMEJ-based CRISPR/Cas9 (mCRISPR) strategy to efficiently generate multiple mutant parasites simultaneously in genes with repetitive sequences. As a proof of principle, we successfully produced various size mutants in the central repeat region of the *Plasmodium yoelii* circumsporozoite surface protein without the use of template DNA. Monitoring mixed parasite populations and individual parasites with different sizes of CSP-CRR showed that the CSP-CRR plays a role in the development of mosquito stages, with severe developmental defects in parasites with large deletions in the repeat region. However, the majority of the *csp* mutant parasite clones grew similarly to the wild type *P. yoelii* 17XL parasite in mice. This study develops a useful technique to efficiently generate mutant parasites with deletions or insertions, and shows that the CSP-CRR plays a role in parasite development in mosquito.

1. Introduction

Malaria parasites are organisms that can survive immune and drug killing by changing their genome sequences or by altering the expression of target molecules. The major mechanisms of genetic changes include nucleotide substitution, deletion/insertion, genetic recombination, or epigenetic modifications. Double-strand breaks (DSBs) of DNA can result in changes in the genome and lead to chromosome translocations, chromosome loss, cell cycle stalling, and cell death (Moynahan and Jasin, 2010). Repair of the DSBs plays an important role in parasite biology and survival. Whereas faithful repair of damaged essential genes is necessary for parasite survival, polymorphisms due to DNA repair error at some specific antigen genes may help the parasite overcome host immune and drug pressures. Three major DSB repair mechanisms have been described in eukaryotic organisms, including non-homologous end joining (NHEJ), homologous recombination (HR), and microhomology-mediated end joining (MMEJ) (Lieber, 2008; McVey and Lee, 2008; Moynahan and Jasin, 2010; Chang et al., 2017). Because no genes encoding enzymes for the NHEJ pathway can be found in the genomes of malaria parasites, the major DSB repair pathway in *Plasmodium* parasites is HR, which has been experimentally verified by numerous HR-based genetic modifications (Lee et al., 2014). An alternative end joining pathway was also described in the human parasite *Plasmodium falciparum* (Kirkman et al., 2014), which is likely to be mediated through MMEJ (Singer et al., 2015). The MMEJ pathway may be responsible for the high rates of small indel mutations in AT-rich malaria genome sequences (Hamilton et al., 2017) and elevated recombination events associated with G-quadruplex motifs (Stanton et al., 2016).

CRISPR/Cas9 is a gene editing technique that has led to great advances in basic research, biotechnology and therapy (Komor et al., 2017). The CRISPR/Cas9 system generally employs a prokaryotic RNA programmable nuclease that can introduce DSBs at a target site identified by sequence-specific guide RNA (sgRNA) (Jinek et al., 2012). This powerful technique has been successfully adapted to edit malaria parasite genomes (Ghorbal et al., 2014; Wagner et al., 2014; Zhang et al., 2014; Lu et al., 2016; Mogollon et al., 2016; Crawford et al., 2017; Zhang et al., 2017). Unfortunately, because malaria parasites lack the critical components of the NHEJ DNA repair pathway such as Ku70/80 and DNA Ligase IV9 (Gardner

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https://doi.org/10.1016/j.ijpara.2019.04.003
0020-7519/

et al., 2002), HR is considered to be the primary DSB repair pathway in this parasite (Straimer et al., 2012). Therefore, CRISPR/Cas9 methods for malaria parasites also require homologous DNA templates flanking the targeted DSB site that are typically provided in a plasmid. The requirement of template DNAs can greatly increase the workload in plasmid construction, particularly when editing a large number of genes or members of a gene family.

The genomes of malaria parasites contain large numbers of simple sequence repeats such as microsatellites in non-coding and minisatellites in coding regions (Su et al., 1999; Gardner et al., 2002). One example is the gene encoding sporozoite surface protein (CSP). The CSP contains two small conserved regions (Regions I and II) and a central repeat region (CRR) containing immunodominant epitopes (Lal et al., 1987). Antibodies specific for the CSP-CRR are important for protective immunity (Sinnis and Nardin, 2002). Indeed, a subunit malaria vaccine candidate composed of the CSP repeats (RTS, S) has been shown to be partially protective, validating the CSP repeats as a vaccine target (Rts et al., 2012; Triller et al., 2017). A total deletion of the CRR of *Plasmodium berghei* CSP leads to defective sporozoites, but appeared to have normal oocysts (Menard et al., 1997; Ferguson et al., 2014). Replacement of the *P. berghei* CSP gene with that of the avian *Plasmodium gallinaceum* parasite also results in non-infective sporozoites (Aldrich et al., 2012). Additionally, CSP is reported to interfere with the import of host nuclear factor kappa beta (NFκB) into the nucleus and to influence the expression of over 1000 host genes (Singh et al., 2007). The highly conserved region I is cleaved by a papain family cysteine protease, which is required for sporozoite invasion into host cells (Coppi et al., 2005). Although CSP is primarily expressed in sporozoites and is essential for sporozoite development (Menard et al., 1997; Ferguson et al., 2014), the effects of CRR size variation on sporozoite development are not clear. This study has two major goals: one is to test the principle of the *Plasmodium* MMEJ pathway in repairing CRISPR/Cas9-mediated DSBs and to develop a simple MMEJ-mediated CRISPR/Cas9 method (mCRISPR) for parasite genome editing. Another goal is to use the method to generate CSP-CRR deletion mutants for study of CSP-CRR functions in parasite development and potentially for vaccine development. We successfully developed and verified the functionality of a mCRISPR method that does not require homologous DNA templates. We then used the mCRISPR to generate *Plasmodium yoelii* CSP-CRR mutants, cloned 10 parasites with different CRRs, and evaluated their development in mice and mosquitoes. The majority of the PyCSP-CRR mutant clones grew similarly to the wild type (WT) 17XL parasite in mice. Parasites with large deletions (172 amino acids (aa)) in the CRR or deletion with a reading frame shift cannot develop into infective sporozoites. The results show that a minimum length of CRR is required for sporozoite development, providing new insights for the role of CSP-CRR in the development of mosquito stages.

2. Materials and methods

2.1. Malaria parasites, mice and mosquitoes

Plasmodium yoelii lethal line 17XL derived from the non-lethal isolate 17X was used in this study (Pattaradilokrat et al., 2008). Female outbred Institute of Cancer Research (ICR) mice and inbred BALB/c mice, 6–8 weeks old, used to maintain and evaluate parasite growth, respectively, were purchased from Xiamen University Laboratory Animal Center or Shanghai Laboratory Animal Center, CAS (SLACCAS), China. A colony of *Anopheles stephensi* mosquitoes (Hor strain) was raised at 23 °C and 75% humidity under a 12:12 light:dark illumination cycle and fed with 5% sucrose solution. All animal experiments were performed in accordance with an approved protocol (#XMULAC20150080) by the Laboratory Animal Management and Ethics Committee of Xiamen University, China.

2.2. Plasmid vector constructs and parasite transfection

Four sgRNAs were designed to target multiple sites in the CRR of the *Pycsp* gene (PlasmoDB accession no. PY17X_0405400, 1284 bp) (Supplementary Fig. S1 and Supplementary Table S1). Cas9-sgRNA plasmids without homologous donor templates were generated based on the pYC vector that contains the gene encoding Cas9 enzyme from *Streptococcus pyogenes* (Zhang et al., 2017). Plasmid without sgRNA which served as a control was generated as well. Transfection and selection of transformed parasites with pyrimethamine were performed as described previously (Nair et al., 2017; Zhang et al., 2017). Briefly, 0.5–1 × 10^7 purified schizonts were mixed with 100 µl of cytomix (120 mM KCl, 0.15 mM $CaCl_2$, 5 mM $MgCl_2$, 25 mM Hepes, 2 mM EGTA, 10 mM KH_2PO_4, pH 7.6) containing 5–10 µg of plasmid DNA in an electroporation cuvette and transfected using the program T-016 in the Nucleofector device (Amaxa). Transfected parasites were immediately injected into a tail vein of a naïve mouse and subsequent selection with pyrimethamine provided in drinking water at a concentration of 7 mg/L from day 2 post-injection. Drug-resistant and transformed parasites usually appear 5–7 days p.i.

Gene deletion and/or insertion events were first detected by PCR amplification using Taq DNA polymerase (#AP111, TransGen Biotech) with primer pairs F1/R1 or F1/R2 flanking the *Pycsp* repeat region (Supplementary Table S1). PCR cycles and set-up were: initial denaturation at 94 °C for 3 min; cycling 94 °C for 20 s, 45 °C for 20 s, 60 °C for 70 s for 30–35 cycles; and a final extension at 60 °C for 3 min. PCR products were separated on 1.5% agarose gels, visualised and photographed on a UV box (Tanon-2500). PCR products, amplified from parasite mixtures (without cloning) or cloned parasites, were sequenced directly by a commercial company (Xiamen Borui Biological Technology Co., Ltd).

2.3. Cloning PCR products in a TA vector and DNA sequencing

To detect deletions or insertions in the CRR among parasite populations after CRISPR/Cas9 editing, DNA from blood stage parasite mixtures transfected with mCRISPR plasmids were amplified, and the PCR products were cloned into a TA vector. The TA plasmids were used to transform *Escherichia coli*. Individual bacterial colonies were picked and grown in Luria-Bertani (LB) broth overnight. Plasmid DNAs were extracted from the bacterial cultures for DNA sequencing.

2.4. Parasite cloning and identification

Limiting dilution cloning of parasites was followed if mutations in the *Pycsp* gene were detected in a transfected parasite population after PCR amplification and/or DNA sequencing. Infected red blood cells (iRBCs) from donor mouse blood were diluted to one parasite per 200 µl of PBS (inoculum size) and were i.v. injected into 20–30 recipient mice. Mice were monitored for the presence of parasites on days 5–9 after the injection. Positive blood samples with a parasitemia of 1–5% were collected for DNA extraction using a phenol/chloroform method and for parasite preservation in a −80 °C refrigerator or liquid nitrogen. Individual clones with CRISPR/Cas9 mediated mutations were identified by PCR assay and confirmed by DNA sequencing.

2.5. Characterisation of asexual blood and mosquito stages

Daily parasitemias of asexual blood stages of WT 17XL and PyCSP-CRR mutant parasites, and host mortality rates, were

evaluated in female BALB/c mice (4–5 mice per single parasite clone). Each mouse was injected i.v. with an inoculum containing approximately 1×10^6 iRBCs. Giemsa-stained thin tail blood films were made daily from day 2 p.i. Parasitemias were monitored and measured by microscopic examination and recorded in an Excel spreadsheet.

For blood feeding of mosquitoes, 200 µl of PBS containing 1×10^6 iRBCs from a donor mouse were injected i.v. into a recipient ICR mouse. Parasitemia and gametocytemia were monitored by thin blood smears and Giemsa stain. Mosquitoes (150–200 females) were fed on the infected mouse for 30 min on days 3–4 p.i. Mosquito midguts were dissected on day 8 after a blood meal and examined for oocysts under a light microscope. Salivary glands were dissected on days 17–19 and harvested for homogenisation; released sporozoites were counted using a hemocytometer. To measure the infectivity of salivary gland sporozoites, naïve mice were infected through mosquito bites.

2.6. Statistics

Pair comparisons were tested using a two tailed *t*-test in Excel or Prism. $P \leq 0.05$ was considered significant.

3. Results

3.1. Generation of PyCSP-CRR mutants using the mCRISPR method

To study the effects of PyCSP-CRR size polymorphism on parasite development, we developed a simple and efficient CRISPR/Cas9 method taking the advantage of the MMEJ DNA repair mechanism recently observed in malaria parasites (Kirkman et al., 2014; Singer et al., 2015). The CSP of 17XL parasite contains two types of tandem repeats [$(QGPGAP)_{25}$ and $(PPQQ)_7$] in the CRR. We designed four guide RNAs (sgRNA, Supplementary Table S1 and

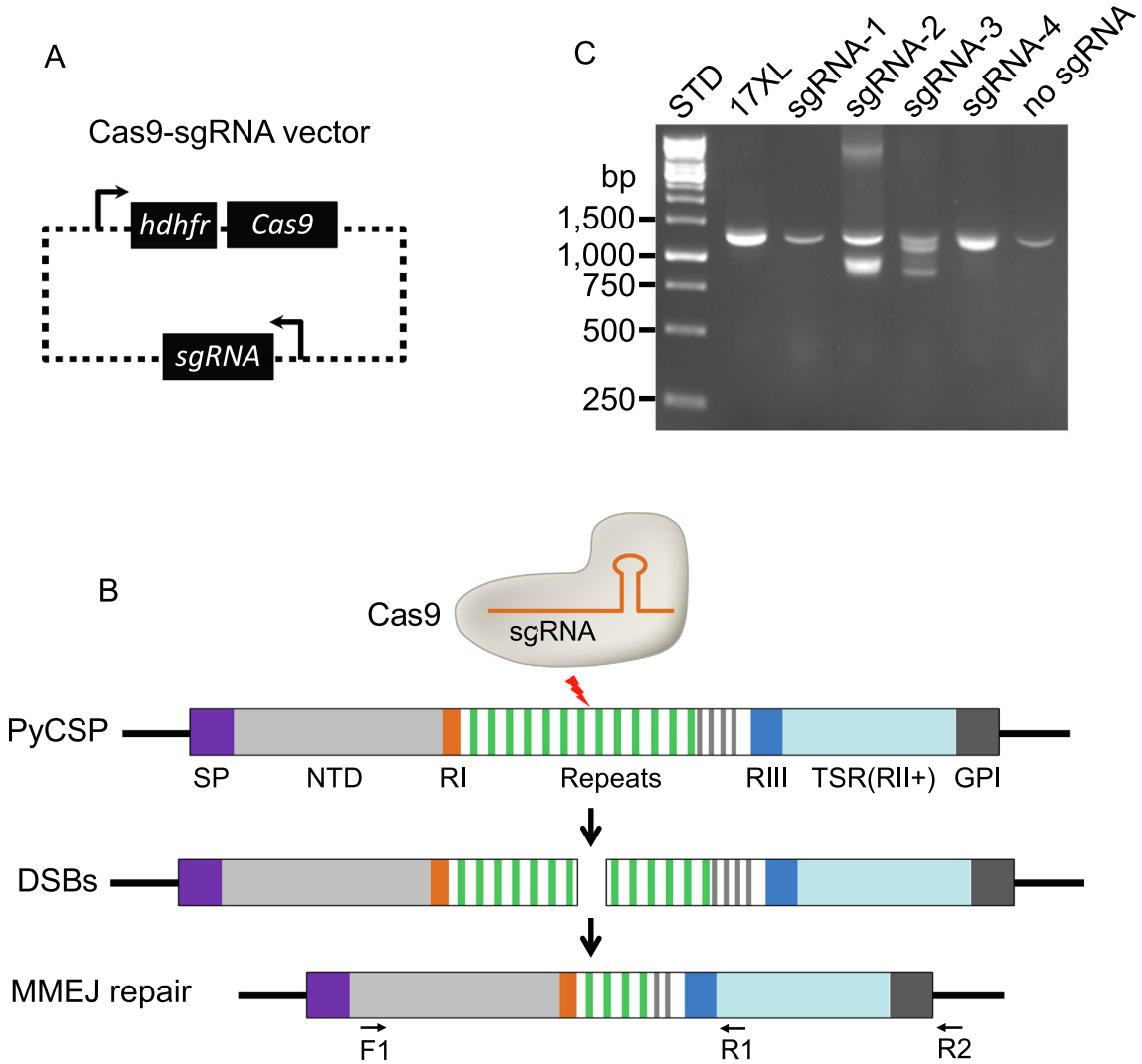


Fig. 1. Demonstration of a microhomology-based CRISPR/Cas9 strategy to generate size mutants of *Plasmodium yoelii* 17XL. (A) A simplified diagram of a transfection plasmid for generation of a double strand break. (B) *Csp* gene structure showing the central repeat region, the conserved regions I and II, and the locations of primers used in PCRs. (C) An agarose gel showing bands of PCR products using primers F1/R2 from day 3 parasite mixtures after MMEJ-based CRISPR/Cas9 (mCRISPR) modification and drug selection. The single guide RNAs used and no sgRNA control are as indicated. PyCSP, *Plasmodium yoelii* circumsporozoite surface protein; DSBs, double-strand breaks; MMEJ, microhomology-mediated end joining; SP, signal peptide; NTD, N-terminal domain; RI, region I; RIII, region III; TSR, thrombospondin type-1 repeat; RII+, region II plus; GPI, glycosylphosphatidylinositol anchor sequence.

Supplementary Fig. S1) from the CRR region and constructed four plasmids that transcribes each of the sgRNAs and the Cas9 enzymes without homologous DNA templates to generate targeted DSBs (Fig. 1A and B). sgRNA-2 is expected to cleave three sites at the CRR because it has three corresponding target sequences, whereas the other three sgRNAs have one cleavage site (Supplementary Fig. S1A). Without the presence of homologous DNA templates, overlapping repetitive sequences may anneal randomly, leading to cleavages of overhanging sequences and sealing of the gaps (Supplementary Fig. S1B) (McVey and Lee, 2008). Target DNA samples were extracted from the 17XL parasite in mouse blood when the parasitemia reached 1–5% after transfection and pyrimethamine selection, and were amplified using primer pairs F1/R1 (or R2) flanking the PyCSP-CRR (Fig. 1B). Multiple bands were detected in parasites transfected with sgRNA-2 and sgRNA-3, whereas a major band similar to that of 17XL WT was observed in the parasites transfected with sgRNA-1 and sgRNA-4 (Fig. 1C). We cloned the amplified DNAs from transfectants with four individual sgRNAs into a TA-vector, transformed bacteria, randomly selected 11 colonies for DNA sequencing, and obtained seven unique sequences with deletion of 234–881 bp and two 17XL WT sequences (Table 1). We also amplified the PyCSP-CRR from the WT 17XL parasite, cloned the PCR product into a TA vector, and sequenced DNA samples from six randomly chosen bacterial colonies. No change in the CRR sequence was observed (data not shown), suggesting that the PyCSP-CRR is relatively stable in *E. coli*. Interestingly, three of the sequences had deletions leading to reading frame shifts, suggesting that a complete functional CSP is not required for blood stage survival (also see Sections 3.3 and 3.4). These results demonstrate that the *P. yoelii* parasite can repair DSBs generated by CRISPR/Cas9 through the MMEJ pathway without the need for homologous DNA templates, although we cannot rule out that some of the cloned mutant sequences were due to deletion during cloning in *E. coli*.

3.2. Fitness of parasites with different CSP-CRR in mice and mosquitoes

To evaluate effects of CSP-CRR size polymorphism on parasite fitness, we transfected the 17XL parasite with plasmids containing genes encoding the Cas9 enzyme and sgRNA-2 or sgRNA-3, respectively. After injection of the transfected parasites into three mice followed by drug selection (7 mg/L of pyrimethamine treatment starting from day 2 p.i.), we extracted DNA from parasite mixtures from day 1 to day 7 post injection. We then PCR-amplified the repetitive region using primers F1/R2 and detected DNA bands on agarose gels. At least six major bands could be detected in the day 1 DNA sample from parasite mixture transfected with sgRNA-2, with the majority of new major bands being smaller than that of the WT band (Fig. 2A). Interestingly, most of the bands became weaker from day 2; and by day 4, a band of ~900 bp (smaller than the WT band) became the dominant band. The WT band disappeared, likely due to continuing cleavage of the WT DNA by Cas9 because it contained the sgRNA sequence, whereas the 900 bp band might not have the sgRNA sequence, thereby avoiding further cleavages. Direct sequencing of the 900 bp band showed a mixture of multiple sequences. Cloning and sequencing of individual DNA from the 900 bp product confirmed the absence of the sgRNA sequences in the full sequences (data not shown), which partially explains the persistence of parasites with the 900 bp DNA. We also performed experiments using sgRNA-2 or sgRNA-3, treated the transfected parasites with pyrimethamine for 7 days starting from day 2 p.i., passed the parasites to naïve mice without further drug treatment, and obtained similar results (Fig. 2B and C). Without drug pressure, traces of the WT band could be detected after day 2, although the ~900 bp products were still the dominant bands. In the sgRNA-3 group, more than six bands were detected day 1 post injection, however, three major bands were retained from day 2 to day 5 (Fig. 2C; mice died earlier without pyrimethamine treatment). These results suggest that parasites with large deletions in the CSP-CRR may not grow as well as parasites with ~900 bp size or WT CSP in mouse blood, although we cannot rule out that negative 'off-target' effects may also contribute to the disappearance of those smaller bands. The results suggest that it is necessary to clone parasites as early as possible in order to obtain mutants with different deletion sizes.

We next fed parasite mixtures to mosquitoes and monitored parasite population dynamics while developing in mosquitoes. We injected a CRISPR/Cas9 (CSP-sg2) transfected parasite mixture (1×10^6 iRBCs) from frozen stock into mice. On day 4 p.i. when gametocytes could be detected in a blood smear, we fed the parasite mixture to mosquitoes and collected DNA samples from day 8, 10, 12 oocysts, day 17 sporozoites, and blood stages after infecting mice with the sporozoites through mosquito bites. Interestingly, the WT band and the original dominant blood stage bands of ~900 bp became the major bands in oocysts (Fig. 2D). After infecting mice, the WT band was the only major band, suggesting that parasites with the WT CSP-CRR can develop better in mosquitoes and/or in the livers of mice than the mutant parasites. In a separate experiment, the WT band did not become the dominant band in the mosquito stages, likely due to alack of WT parasite; instead, the ~900 bp bands remained the dominant bands (Fig. 2E). The results suggest that some parasites with a mutant CSP-CRR may not develop well, or at all, in mosquito and/or in the liver stages.

Table 1
Plasmodium yoelii circumsporozoite surface protein deletion clones obtained after MMEJ-based CRISPR/Cas9 editing and TA cloning.

Clone[a]	sgRNA	# of bp	# bp del.	# of AA	# AA del.
17XL		1284	/	427	/
C1-4	sg1	403	881	33[b]	394
C1-21	sg1	1284	0	427	0
C2-7	sg2	888	396	295	132
C2-8	sg2	1284	0	427	0
C2-9	sg2	1050	234	349	78
C3-31	sg3	771	513	256	171
C4-36	sg4	1032	252	343	84
C4-37	sg4	404	880	74[b]	353
C4-39	sg4	530	754	98[b]	329

sgRNA, single guide RNA used in CRISPR editing.
MMEJ, microhomology-mediated end joining.
bp del., number of bp deleted or inserted, compared with that of wild type.
of AA, number of amino acids in the amplified segments.
AA del., number of amino acids deleted or inserted.
[a] Bacterial colony after TA cloning (clone the PCR product into a vector with 3′-T overhangs).
[b] Mutation in open reading frame caused premature termination.

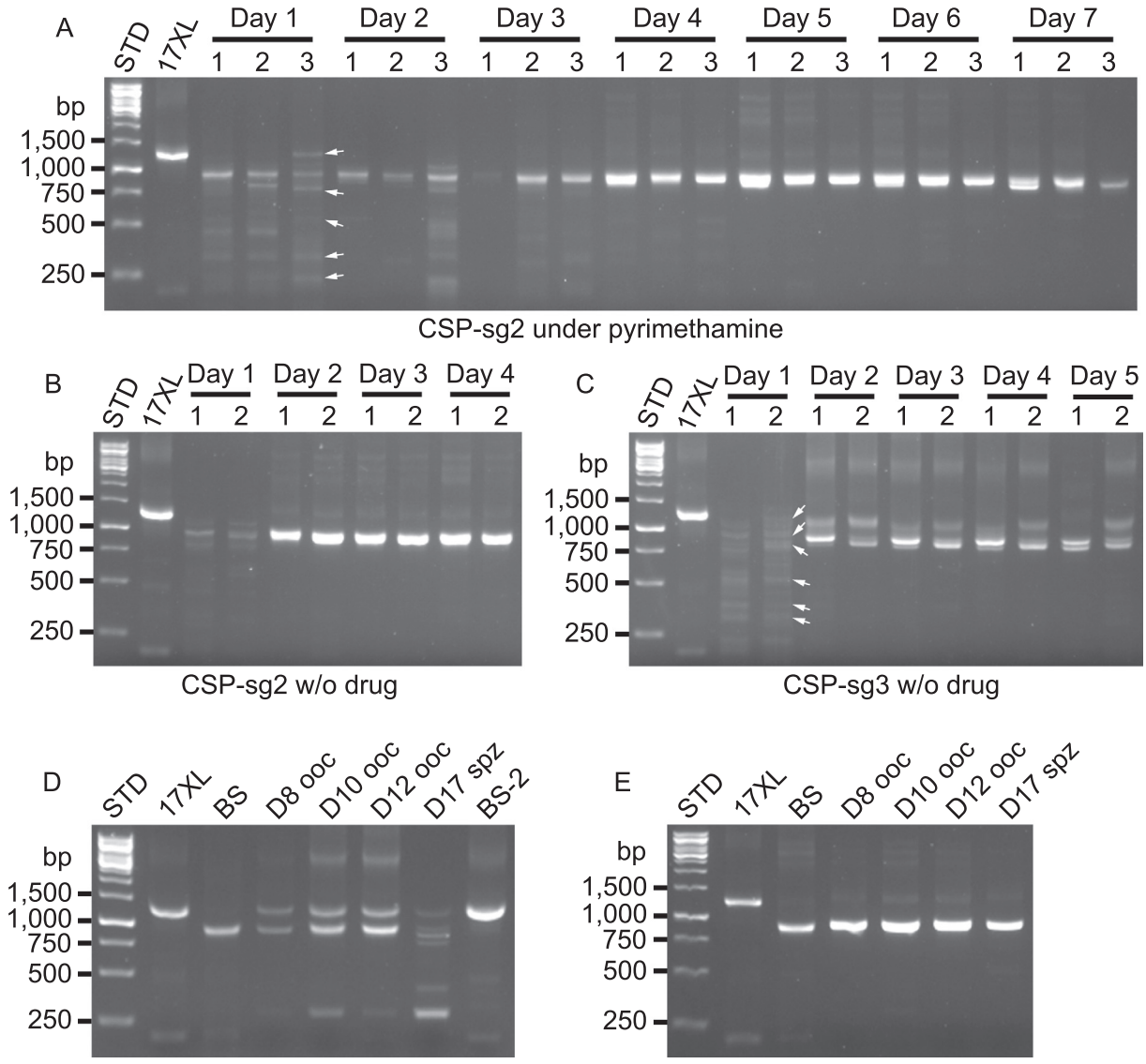


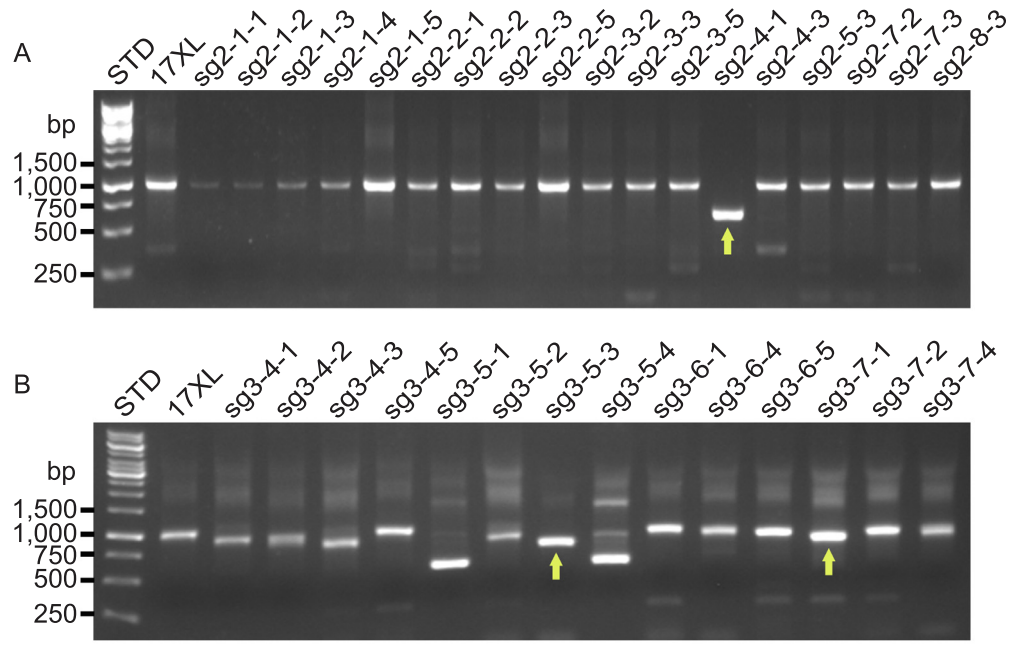
Fig. 2. Selection of *Plasmodium yoelii* circumsporozoite surface protein gene central repeat mutant alleles during development in the blood and in mosquito. (A) Various *Pycsp* central repeat alleles amplified from day 1–7 parasite mixtures after transfection and drug selection. (B and C) Various *Pycsp* central repeat alleles amplified from blood stages without drug selection after passing the parasites to naïve mice. (B) Transfected with single guide RNA (sgRNA)-2; (C) transfected with sgRNA-3. (D) *Pycsp* central repeat alleles amplified from blood stages (BS), day 8 oocysts (D8 ooc), day 10 oocysts (D10 ooc), day 12 oocysts (D12 ooc), day 17 sporozoites (D17 spz) or blood stages after infection with sporozoites (BS-2). (E) A similar experiment as in (D) with sporozoites that had a *Pycsp* central repeat of ~900 bp and failed to infect mice. Amplification products in (A–E) were obtained using primer pair F1/R2.

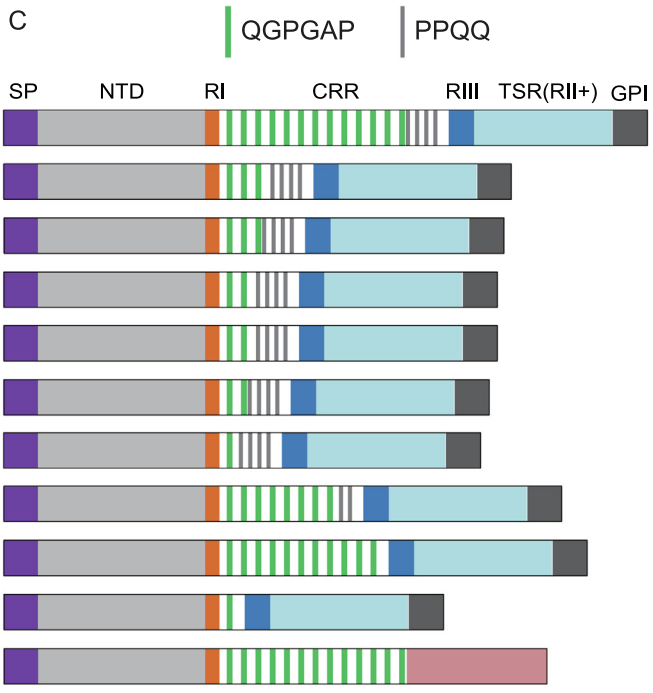
3.3. Cloning parasites with different csp repeat lengths

To further characterize the functional roles of PyCSP-CRR in parasite development, we attempted to clone parasites with different lengths of CRR. We performed limiting dilution cloning by injecting ~1 (in 100 µl of PBS) parasite transfected with the sgRNA-2 or sgRNA-3 CRISPR/Cas9 plasmid into 120 mice in five independent experiments (Supplementary Table S2). Amplification of DNA samples from these parasites on day 7 p.i. showed that the majority of the parasite clones carried the WT CSP-CRR, except one clone with a smaller repeat region (clone sg2-4-1, Fig. 3A). There were also several clones (sg2-2-1, sg2-2-2, sg2-3-5, sg2-4-3, and sg2-7-3) that might still have mixed parasites with a dominant WT parasite population and a minor population with smaller CRR (Fig. 3A). Because these parasites were cloned from frozen stock with a parasitemia of >1%, the parasite with WT repeat might have outgrown the other parasites with mutant CRRs during rounds of growth in mice. To improve the efficiency in obtaining CSP-CRR mutant parasites, we transfected mice with the mCRISPR plasmids again and cloned parasites directly as soon as parasites could be detected in a blood smear (<0.1%). We obtained more parasite clones with different CSP repeat lengths on agarose gel (Fig. 3B). After DNA sequencing of PCR products from 25 cloned parasites of different transfections, we obtained nine independent parasite clones with different sizes of the PyCSP-CRRs, carrying deletions from 46 aa to 172 aa (Fig. 3C, Supplementary Figs. S2 and S3). One of the clones (sg3-4-4) had changes leading to disruption of open reading frame (ORF) and a truncated protein. These cloning results confirm that parasites with different CSP-CRRs can still grow in mouse blood. In the experiments without cloning, the parasite with WT CSP could be affected by the presence of CRISPR/Cas9 activity, and therefore may not become the dominant population.

3.4. Effects of different CSP-CRRs on blood stage growth

We next infected mice with 10 parasite clones (clone sg2-2-12, sg2-2-21, sg2-2-22, sg2-2-24, sg2-4-1, sg2-4-2, sg3-2-63, sg3-4-4, sg3-5-3, sg3-7-1, and WT 17XL) carrying nine different





	No. of CSP repeat units		
	AA del.	QGPGAP	PPQQ
17XL	/	25	7
sg2-2-22	-114	6	7
sg2-2-12	-120	5	7
sg2-4-1	-126	4	7
sg2-2-24	-126	4	7
sg2-2-21	-132	3	7
sg2-4-2	-138	2	7
sg3-5-3	-66	16	4
sg3-7-1	-46	22	0
sg3-2-63	-172	1	0
sg3-4-4	-79	25	0

Fig. 3. Cloning and verification of parasites with different sizes of *Plasmodium yoelii* circumsporozoite surface protein gene central repeat. (A) PCR products of parasites cloned from mouse blood injected with MMEJ-based CRISPR/Cas9 (mCRISPR) modified parasite mixtures. Blood from the mouse receiving the transfected parasites was first frozen, and a stock of 100 µl was thawed and injected into a second mouse. Day 3 blood was collected for limiting dilution cloning. Parasite names are above the gel eg sg2-1-1 indicates clone 1-1 from the mixture transfected with single guide RNA (sgRNA)-2. (B) Similar parasite cloning and PCR amplification as in (A), except that the parasites were cloned the at a low parasitemia (<1%) directly from the mouse receiving electroporated parasites. (C) Summary of parasite clones with different numbers of amino acids deleted in the *Pycsp* central repeat region (CRR). Primer pair F1/R1 was used to amplify the products in (A) and (B). SP, signal peptide; NTD, N-terminal domain; RI, region I; RIII, region III; TSR, thrombospondin type-1 repeat; RII+, region II plus; GPI, glycosylphosphatidylinositol anchor sequence.

PyCSP-CRRs (sg2-2-24 and sg2-4-1both have 126 aa deletion; Fig. 3) to evaluate effects of individual CRR mutants on parasite development. Eight of the 10 mutant parasites grew similarly to the WT parasite, except clones sg2-2-22 (114 aa deletion) and sg3-7-1 (46 aa deletion) that had significantly lower parasitemias than those of the WT parasite on days 3–5 p.i. (Fig. 4A and B). Mice infected with these two parasites also survived longer than those infected with WT and other mutant parasites (Fig. 4C and D; euthanized on day 20). To evaluate the target site specificity, we searched the 17X genome for sequences homologous to the *Pycsp* sgRNA sequences we used. We found one and three potential off-target sites for sgRNA-2 and sgRNA-3, respectively (Supplementary Table S4). All of the four off-target sequences had ≥3 nucleotide mismatches. DNA sequencing of PCR-amplified products of the potential off-target sites detected no mutations at these sites (Supplementary Table S4). However, we cannot rule out that the changes in growth for these two parasites were caused by other 'off-target' effects or unknown mutations in the genome because the parasitemias of

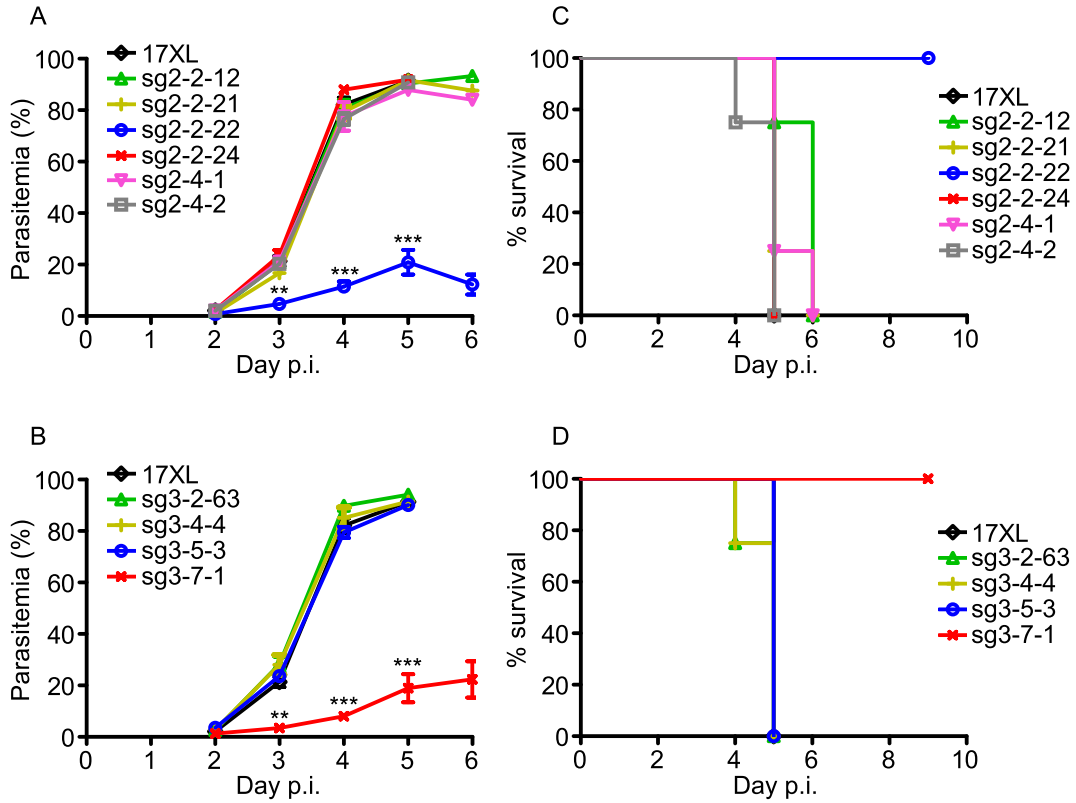


Fig. 4. Parasitemia and host survival rates of wild type 17XL and *Plasmodium yoelii* circumsporozoite surface protein gene repeat mutants. Individual parasite clones (1×10^6) were injected i.v., and blood smears were prepared and counted as described in Section 2. (A and B) Parasitemias of BALB/c mice infected with WT 17XL and six single guide RNA (sgRNA)-2 *Pycsp* central repeat mutant parasites (A) or four sgRNA-3 *Pycsp* central repeat mutant parasites (B). (C and D) Survival curves of 17XL and the *Pycsp* central repeat mutant parasites. Means and standard errors were calculated from four or five mice for each parasite clone; unpaired two-tailed *t*-test, $^{**}P < 0.01$; $^{***}P < 0.001$.

the parasites with larger deletions or even disruption of ORFs (sg3-4-4) were not affected by the deletions. These results appear to contradict the observations from those of uncloned parasite mixtures, which showed that a parasite with WT or a PyCSP-CRR slightly smaller than that of WT parasites grew better than parasites with other mutant CRRs. One possible explanation is that the growth advantage of the parasites can only be observed in a mixture directly competing for survival in the same mouse. However, the mechanism for the disappearance of some small PyCSP-CRR bands in transfection mixture over time requires further investigation.

3.5. Development of PyCSP-CRR mutants in mosquitoes

We also infected mosquitoes with the 10 individual CSP CRR mutants, counted day 8 oocysts and day 17 salivary gland sporozoites, and infected mice again through mosquito bites. Large variations in oocyst counts were observed between individual feedings, depending on each batch of mosquitoes. No difference in day 8 oocyst counts was observed when data from different feedings were combined (Fig. 5A). A significant reduction in sporozoite infectivity was found for sg2-2-12 (120 aa deletion) and sg2-4-2 (138 aa

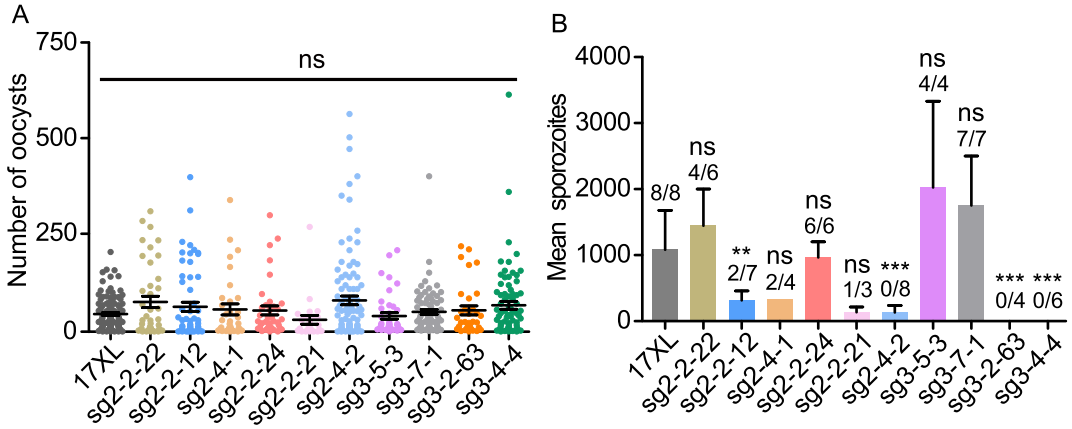


Fig. 5. Oocyst and sporozoite counts from mosquitoes infected with 10 *Plasmodium yoelii* circumsporozoite surface protein gene central repeat mutants. (A) Day 8 oocyst counts for wild type 17XL and the *Pycsp* central repeat mutants. The middle level bars are mean oocyst counts, and the lower and upper bars are standard errors, respectively. (B) Day 17 salivary gland sporozoite counts for wild type 17XL and the *Pycsp* central repeat mutants. The numbers above each bar are the numbers of infected mice/numbers of challenged mice in each group. Means and standard errors were calculated from three to eight mice for each parasite clone; unpaired two-tailed *t*-test comparing sporozoite infectivity (percentage of mice infected) from each individual clone and 17XL, $^{**}P < 0.01$; $^{***}P < 0.001$; ns, no significance.

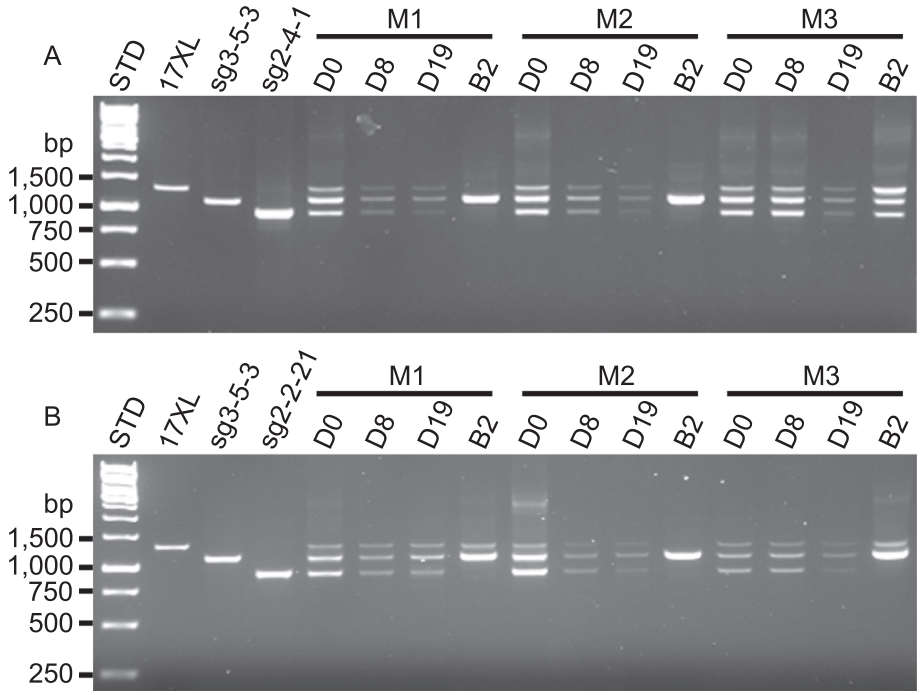


Fig. 6. Growth dynamics of *Plasmodium yoelii* circumsporozoite surface protein gene central repeat mutant clones in mosquitoes over time. 17XL, sg3-5-3 and sg2-4-1 (or sg2-2-21) parasites were counted, mixed at approximately at a 1:1:1 ratio and injected into a mouse. Mosquitoes fed on the mouse on days 3–4 p.i. DNA from parasites before injection (D0), day 8 oocysts (D8), day 19 salivary gland sporozoites (D19), and blood of infected mice after mosquito bites (B2) were extracted and amplified for 27 cycles (for B2, 29 cycles to increase signals). (A) Amplified PCR products from 17XL/sg3-5-3/sg2-4-1 parasite mixture. (B) Similar experiments as in (A) from 17XL/sg3-5-3/sg2-2-21 parasite mixture. M1-M3 indicates mosquitoes fed on three individual mice. Each DNA sample was extracted from 20 to 30 dissected salivary glands.

deletion) parasites after infection of mice through mosquito bites (Fig. 5B). Additionally, the clone with a 172 aa deletion (sg3-2-63) and the one with a reading frame shift (sg3-4-4) were not able to produce mature sporozoites to infect mice (Fig. 5B and Supplementary Table S3). These results show that a minimum length of CSP-CRR is required for the development of infective sporozoites.

We next fed *A. stephensi* mosquitoes with the parasite mixtures having different *csp* repeat lengths to evaluate the impact of *csp* repeat variations on parasite growth in mosquitoes (Fig. 6). For the first mixture, all three parasites developed to mature sporozoites, but the sg3-5-3 band became dominant in two of the three mice after mosquito bites (Fig. 6A). Similarly, sg3-5-3 became dominant and sg2-2-21 could not be detected in all three mice for the second mixture (Fig. 6B), suggesting that a 132 aa deletion might affect parasite migration to, or development in, the liver.

4. Discussion

This study develops a MMEJ-based CRISPR/Cas9 gene editing strategy to generate size polymorphisms in PyCSP-CRR. Various CRISPR/Cas9 methods have been developed for editing malaria parasite genomes; however, they all require the supplies of homologous DNA templates (Ghorbal et al., 2014; Wagner et al., 2014; Zhang et al., 2014, 2017; Lu et al., 2016; Mogollon et al., 2016; Crawford et al., 2017) due to the absence of the NHEJ machinery in the parasite genomes (Gardner et al., 2002). The discovery of the alternative MMEJ mechanism in *P. falciparum* provides an opportunity for development of an efficient method to generate a large number of size mutants in genes with repetitive sequences (Kirkman et al., 2014; Singer et al., 2015). MMEJ-based CRISPR methods have been developed and tested in various organisms such as the pathogenic fungus *Aspergillus fumigatus*, *Trypanosoma cruzi*, and HEK293T cells (Nakade et al., 2014; Peng et al., 2014; Zhang et al., 2016). In *T. cruzi*, it was shown that DSB repair by HR was much more efficient than MMEJ if a template for DNA repair was provided (Peng et al., 2014). Additionally, the MMEJ-based CRISPR technique was also successfully employed to knock down the expression of members of a gene family in *T. cruzi*, suggesting an important tool for studying gene families. Many malaria parasites have large numbers of repetitive sequences and gene families (Cunningham et al., 2010); genetic manipulation of gene families and repetitive sequences in the parasites has been a difficult task. The mCRISPR strategy we demonstrated may be applied to manipulate and study malaria gene families in a way similar to techniques used for random mutagenesis, although more work needs to be done to show that this technique is a practical method for modification of gene families in malaria parasites.

MMEJ repairs of DSBs requires 5–25 bp microhomologous sequences that align at DSB ends and generally results in deletions (McVey and Lee, 2008). Our data from repair of DSBs in the PyCSP-CRR also showed that the majority of the DNA bands amplified from parasite mixtures and from cloned parasite mutants had deletions. We obtained cloned parasites with deletion lengths ranging from 138 to 516 bp. However, we also observed DNA bands larger than that of WT from PCR amplification of mCRISPR modified parasite mixtures and obtained cloned sequences that were larger than the WT sequence, showing that both deletion and insertion mutants can be generated using mCRISPR. These results suggest that mCRISPR could be an important tool for generating genetic polymorphisms in genes with repetitive sequences or gene families of malaria parasites. For MMEJ-based repair, the sgRNAs are designed to target repetitive sequences, and one sgRNA can potentially target more than one site in the repeat region. Different sgRNAs may have different efficiencies in cutting the DNA. sgRNA-2 has three targeting sites in the CSP-CRR region and could be more efficient than other sgRNAs in mediating DNA cleavage. The location of a sgRNA may also affect cleavage efficiency. sgRNA-2 and sgRNA-3 are located more centrally than sgRNA-1 and sgRNA-4, and appear to be more efficient in cutting the DNA. Differences in cleavage

efficiency may influence the frequency of parasites with WT CSP-CRR regions after transfection. Additionally, the lack of drug pressure during development in the mosquito may increase the frequency of the WT parasite if the WT CSP-CRR is not completely cleaved during blood stage development. There will be variations in deletion/insertion sizes and locations between individual parasites, depending on which and how many sequences are cleaved.

The repetitive region of the CSP has been shown to encode protective epitopes, to contribute to modulation of host immune responses, and to be critical for sporozoite development and parasite transmission (Ferguson et al., 2014; Triller et al., 2017). Although isogenic parasite clones without the *csp* repeat or replacement of the *csp* gene from a different malaria species have been generated using the traditional homologous replacement (Persson et al., 2002; Aldrich et al., 2012; Espinosa et al., 2013; Ferguson et al., 2014), the effect of the size polymorphism of CSP-CRR on parasite development has not been carefully investigated. Different parasite strains often have size polymorphisms in the CSP-CRR; however, field isolates or strains often have genetic differences in other parts of genomes, making it difficult to evaluate the roles of different lengths of CSP-CRR in parasite development and in modulation of host immune responses. The mCRISPR can be a good strategy for generating parasite clones that have different lengths of CSP-CRR with the same genomic background to test the effects of CSP-CRR length variation on parasite development in mosquitoes. We obtained two PyCSP-CRR mutant parasite clones that could not develop into infective sporozoites in mosquitoes, with one clone having an interruption in the coding region and the other having a large deletion (172 aa). It is understandable that parasites with a large deletion or disruption of the ORF of CSP would suffer more severe consequences. The second largest deletion clone, sg2-4-2, had a 138 aa deletion. This parasite still developed into salivary gland sporozoites in two of four batches, but was not able to infect mice after mosquito bites. These results suggest that a minimum CSP-CRR length is required for development in mosquitoes. It would be interesting to obtain additional clones with deletions which may still produce infective sporozoites, but might not be able to develop in the liver stage.

Additionally, the observations of selection for WT and/or the ~900 bp band during blood stage development suggest that either the PyCSP-CRR plays a role in blood stage development or that differential immune responses are triggered by PyCSP with different repeat lengths. However, since the genomes of the mutant parasites were not sequenced to detect potential unknown off-targets or other unknown mutations, it is unclear whether loci other than CSP were affected. Further investigations are necessary to examine these possibilities.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (#81572017, and #81220108019), by Fundamental Research Funds for the Central Universities of China (20720160057), by Project 111 of the State Bureau of Foreign Experts and Ministry of Education of China (B06016) (to J.L.), and by the Division of Intramural Research at the National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), USA (to X-z.S.). The authors thank Brian Brown, NIH Library Writing Center, for manuscript editing assistance. The authors declare there are no competing financial interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2019.04.003.

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