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CRISPR/Cas9 Mutagenesis in *Phlebotomus papatasi*: the Immune Deficiency Pathway Impacts Vector Competence for *Leishmania major*

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ABSTRACT Sand flies are the natural vectors for the *Leishmania* species that produce a spectrum of diseases in their mammalian hosts, including humans. Studies of sand fly/*Leishmania* interactions have been limited by the absence of genome editing techniques applicable to these insects. In this report, we adapted CRISPR (clustered regularly interspaced palindromic repeat)/Cas9 (CRISPR-associated protein 9) technology to the *Phlebotomus papatasi* sand fly, a natural vector for *Leishmania major*, targeting the sand fly immune deficiency (IMD) pathway in order to decipher its contribution to vector competence. We established a protocol for transformation in *P. papatasi* and were able to generate transmissible *null* mutant alleles for Relish (Rel), the only transcription factor of the IMD pathway. While the maintenance of a homozygous mutant stock was severely compromised, we were able to establish in an early generation their greater susceptibility to infection with *L. major*. Flies carrying different heterozygous mutant alleles variably displayed a more permissive phenotype, presenting higher loads of parasites or greater numbers of infective-stage promastigotes. Together, our data show (i) the successful adaptation of the CRISPR/Cas9 technology to sand flies and (ii) the impact of the sand fly immune response on vector competence for *Leishmania* parasites.

IMPORTANCE Sand flies are the natural vectors of *Leishmania* parasites. Studies of sand fly/*Leishmania* interactions have been limited by the lack of successful genomic manipulation of these insects. This paper shows the first example of successful targeted mutagenesis in sand flies via adaptation of the CRISPR/Cas9 editing technique. We generated transmissible *null* mutant alleles of *relish*, a gene known to be essential for the control of immune response in other insects. In addition to the expected higher level of susceptibility to bacteria, the mutant flies presented higher loads of parasites when infected with *L. major*, showing that the sand fly immune response impacts its vector competence for this pathogen.

KEYWORDS CRISPR/Cas9, IMD pathway, Leishmania, sand fly

Leishmania species are responsible for a spectrum of diseases in their mammalian hosts, including humans, ranging from localized cutaneous lesions to fatal visceral disease. Their transmission is achieved via hematophagous insect vectors called sand flies. Parameters intrinsic to these insects, such as expression by midgut cells of receptors recognizing surface components of *Leishmania*, influence their ability to carry and transmit these parasites (1, 2). Among those intrinsic factors, the role of the fly's immune response is of particular interest. The current knowledge about immune responses in insects is based largely on work in *Drosophila* (for reviews, see references 3 and 4) that described two signaling pathways, the Toll and the immune deficiency (IMD) pathways, that are crucial for the insect immune defense against bacteria and fungi. Both pathways are activated by the detection of microbial surface molecules,

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leading to the upregulation of genes encoding antimicrobial peptides (AMPs) that are directly toxic to the pathogen.

Roles for insect immunity in controlling their transmission of infectious pathogens have been reported previously, including transmission of dengue virus by *Aedes aegypti* mosquitos (5–7), of African trypanosomes by the tsetse fly *Glossina morsitans* (8), and of malaria parasites by *Anopheles gambiae* (9, 10). Concerning sand flies, a previous study showed that both the Toll and IMD pathways are activated by yeast, bacteria, and *Leishmania* in cultured *Lutzomyia longipalpis* sand fly cells (11). A defensin AMP was produced in *Phlebotomus duboscqi* flies after challenge by injected bacteria or feeding with bacteria or *L. major* and also presented an antiparasitic action *in vitro* (12). In *Lutzomyia longipalpis* sand flies, the expression of a closely related defensin was shown to be modulated by oral infection with bacteria but did not present significant variation after administration of a blood meal containing *L. mexicana* (13). Finally, silencing of the IMD-negative regulator Caspar by the use of RNA interference (RNAi) led to a reduction of *Leishmania* numbers in the infected midgut (14). Thus, there is evidence to suggest that the sand fly immune response could control its permissiveness with respect to *Leishmania*.

More conclusive evidence to support the idea of a role for innate immune pathways in regulating sand fly vector competence for *Leishmania* has been lacking, largely due to the absence of genome editing techniques adapted for use in these insects. No examples of sand fly mutagenesis have been published so far, and gene downregulation by small interfering RNA (siRNA) is possible but technically challenging, as it requires microinjection and the survival of adult females (14–17). In addition, gene silencing by siRNA can lead to only a partial loss of function which cannot be transmitted from generation to generation. The emergence of CRISPR (clustered regularly interspaced palindromic repeat)/Cas9 (CRISPR-associated protein 9) technology opened new possibilities of genome editing, in particular, in non-model organisms such as sand flies. Present in a wide variety of bacteria and archaea, the CRISPR/Cas9 system was discovered as an adaptative immune system in prokaryotes as a defense against bacteriophages (18, 19). The CRISPR/Cas9 system is based on two elements: (i) a single guide RNA (sgRNA), which is a small RNA containing 17 to 20 bases of complementarity to a specific genomic locus, and (ii) the Cas9 protein, which is able to bind the sgRNA and to create a double-strand DNA (dsDNA) break in the genomic DNA where the sgRNA associates with its complementary sequence. The double-stranded DNA (dsDNA) break created by the Cas9 nuclease can then be repaired either by nonhomologous end joining (NHEJ) or by homology-directed repair (HDR) (for a review, see reference 20). NHEJ involves a simple closure of the break but frequently leads to small insertion/deletion events, whereas HDR uses the presence of a donor DNA molecule sharing homology with the target DNA as a template for repair. Depending on the nature of the eventual donor template used, CRISPR/Cas9 can be used to achieve targeted mutations or more-complex genome editing strategies, such as knock-ins or the generation of expression reporters.

CRISPR/Cas9 was adapted to *Drosophila* in 2014 (21) and is now part of the genetic manipulation toolbox in this classical insect model. CRISPR/Cas9 genome-engineering has also been performed with success in *A. gambiae* and *A. aegypti* mosquitoes (22–25), in the *Tribolium castaneum* red flour beetle (26), and in the *Bombyx mori* silkworm (27). To our knowledge, no successful CRISPR/Cas9 mutagenesis in sand flies has been reported. Sand fly embryos were injected with a CRISPR/Cas9 mix targeting the gene encoding Yellow, but no adults carrying the mutation were produced (28). In this study, we adapted the CRISPR/Cas9 technique to *Phlebotomus papatasi* sand flies, a natural vector for *L. major*. We chose to focus on the IMD pathway rather than the Toll pathway due to the well-characterized role of Toll in dorsoventral axis formation in *Drosophila* (29, 30). The IMD pathway consists of a phosphorylation cascade triggered by the recognition of bacterial PAMPs (pathogen-associated molecular patterns), leading to activation by cleavage of the NF-κB transcription factor Relish (Rel). Activated Rel in turn activates the transcription of its target genes, in particular, those encoding AMPs.

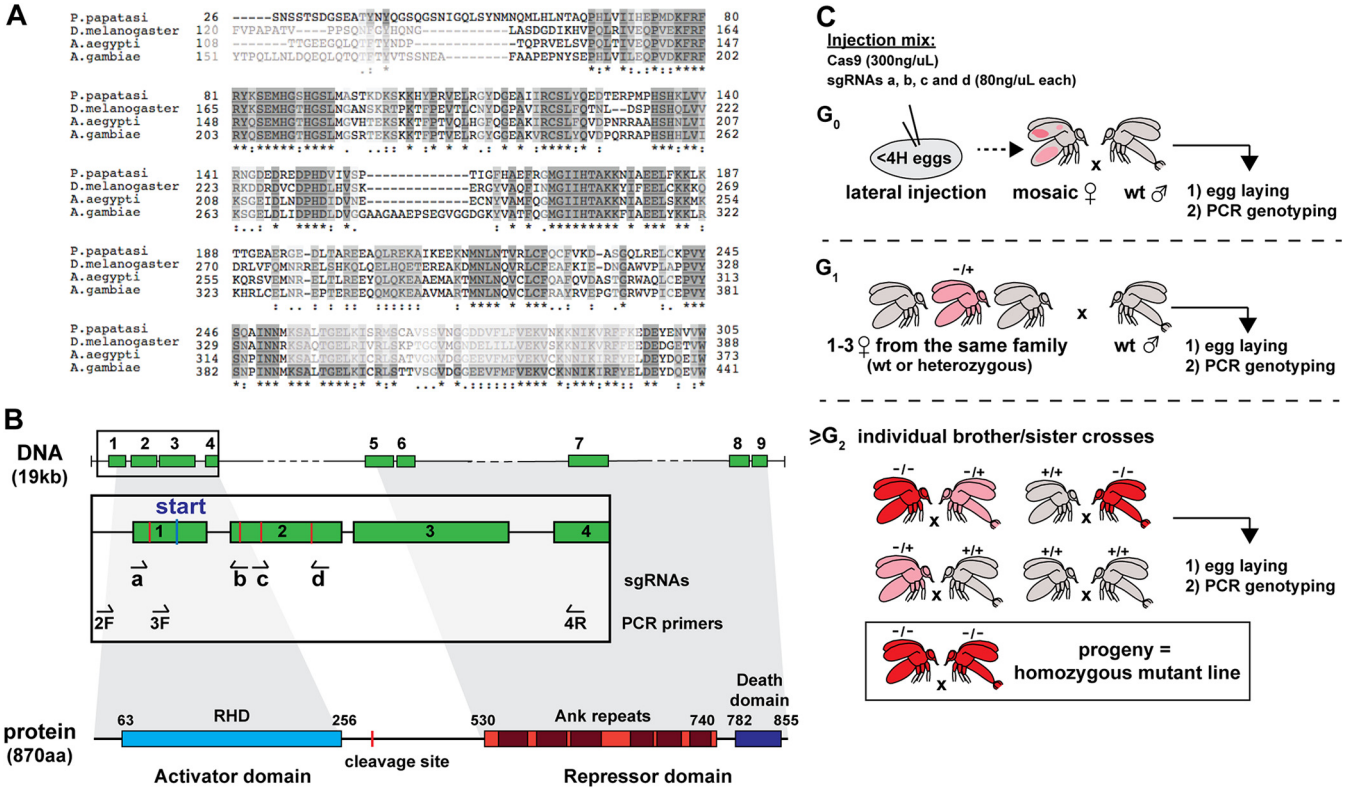


FIG 1 Experimental strategy of *rel* mutagenesis in *P. papatasi*. (A) Alignment showing the high evolutive conservation of the activator RHD from *P. papatasi*, *D. melanogaster*, *A. aegypti*, and *A. gambiae*. (B) Schematic representation of the *P. papatasi rel* gene and Rel protein. The positions of the sgRNAs designed for knocking out *rel* and of the primers designed for screening mutant alleles are indicated by the arrows. (C) Schematic representation of the experimental strategy to isolate mutant alleles and establish homozygous mutant stocks.

The choice of *rel* as a target for mutagenesis was supported by the fact that it is the only known transcription factor for the IMD pathway, which should enable avoidance of redundancy effects, as well as the fact that *rel null* mutants are viable in *Drosophila* despite presenting a clear immune response defect when exposed to bacteria (31).

We present here the strategy successfully adopted to generate and maintain *rel* mutant alleles in *P. papatasi* sand flies by the use of CRISPR/Cas9 technology. *rel* mutant flies presented higher bacterial loads in their gut microbiota and increased numbers of parasites after infection by *L. major*, providing the clearest evidence to date that the sand fly immune response influences vector-parasite compatibilities in leishmaniasis.

RESULTS

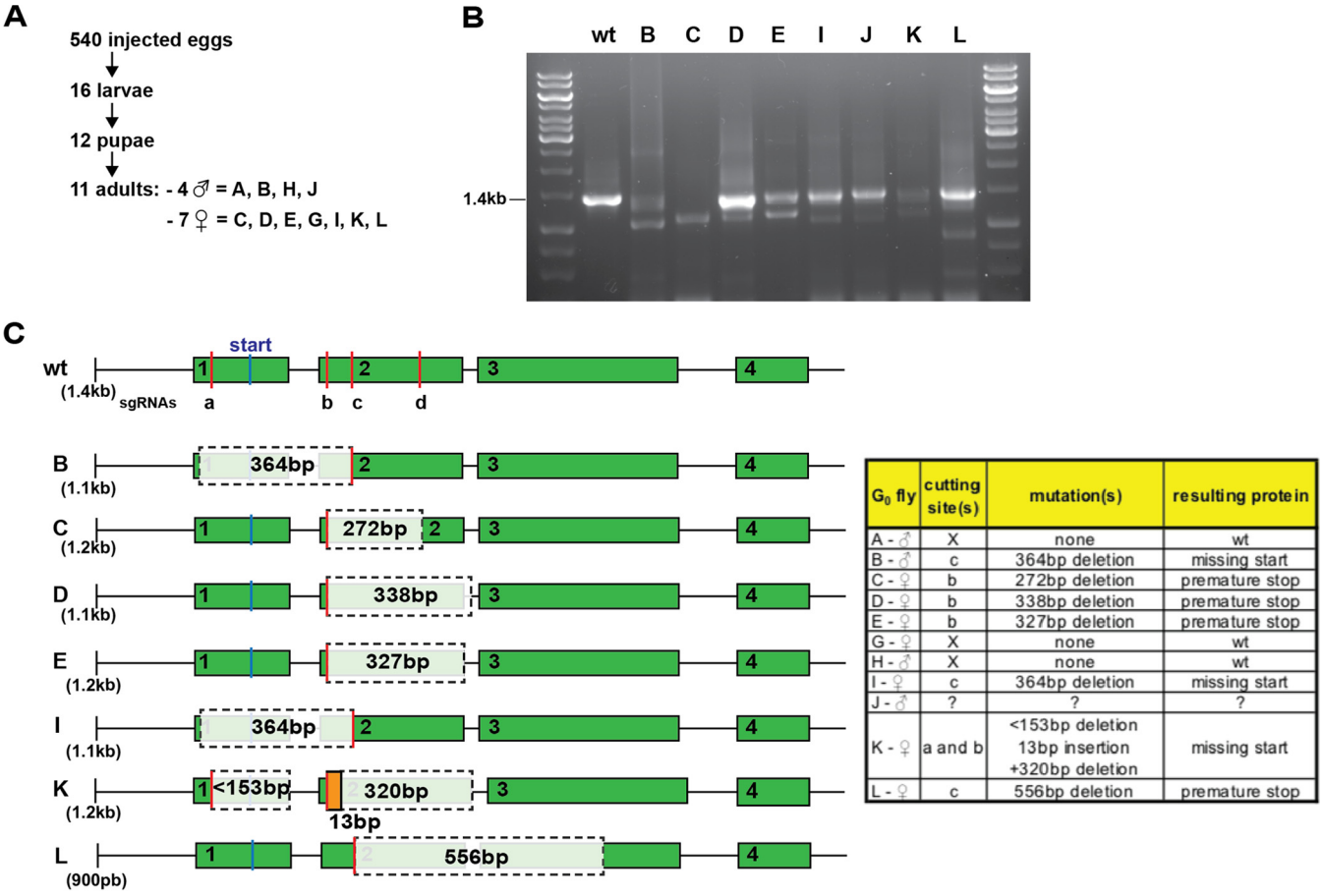
Experimental strategy to knock out *relish* in *P. papatasi* using CRISPR/Cas9. The sequences of the *relish* gene (*rel*) and the Relish protein (Rel) of *P. papatasi* (PPAI012820) are available, and the protein sequence shows high conservation with its homologues in other insect species, including *Drosophila melanogaster* (FBgn0014018), *A. aegypti* (AAEL007624), and *A. gambiae* (Fig. 1A). The 19-kb *P. papatasi rel* gene contains 9 exons and encodes an 870-amino-acid (aa) transcription factor comprised of two distinct predictive domains: an N-terminal activator domain, also called the Rel homology domain (RHD), able to bind the DNA of target genes, and a C-terminal repressor domain, containing several ankyrin repeats and a death domain (Fig. 1B). Exons 1 to 4 code for the activator domain, whereas exons 5 to 9 code for the repressor domain. Exons 4 and 5, exons 6 and 7, and exons 7 and 8 are separated by large intronic regions for which the genomic sequences are currently not available.

Our strategy to generate a total-loss-of-function allele of *rel* was to target the genomic region encoding the most N-terminal part of the protein. Apart from the presence of a few small genomic variations without consequences to the encoded

protein, the sequence of the genomic region consisting of exons 1 to 4 from our colonized flies aligned perfectly to the reference sequence (see Fig. S1 in the supplemental material). Using CRISPR/Cas9 ChopChop software, we designed four sgRNAs within *rel* exons 1 and 2: one (sgRNA a) in exon 1, before the start codon, and three (sgRNAs b, c and d) in exon 2 (Fig. 1B; see also Fig. S2). Our strategy to generate a *rel* null mutant line was adapted from a study in *Aedes aegypti* (24) as follows. The injection mix, composed of Cas9 recombinant protein mixed with sgRNAs a, b, c and d, is injected into ≤4-h embryos (G_0) (Fig. 1C). The G_0 females developing from these embryos, supposedly mosaic for *rel* alleles, are crossed with wild-type (wt) males, allowed to lay eggs, and later genotyped. In order to rapidly screen for the presence of mutant alleles, we designed a simple PCR assay amplifying a 1.4-kb product in wt *P. papatasi* flies (PCR Rel2F-4R) (Fig. 1B; see also Fig. S3). Only the tubes containing a G_0 fly showing one or more additional PCR products are retained. The flies from the next generations are crossed either with wt males (G_1 females) or individually between brothers and sisters (from G_2), allowed to lay eggs, and then subjected to PCR screening for mutations. The last step is repeated until homozygous mutant males and females are obtained, establishing a *null* mutant line (Fig. 1C).

Generation of mutant alleles in G_0 injected individuals. After several tests of injection and rearing conditions, our *rel* CRISPR injection mixture was injected into 540 freshly laid *P. papatasi* eggs, of which only 11 (4 males and 7 females) reached adulthood (Fig. 2A). Given the low number of G_0 adults obtained, we chose to cross both G_0 males and females individually with wt males or virgin females instead of using only the G_0 females as initially planned. The females from each cross were allowed to lay eggs (corresponding to generation G_1), and the G_0 adults were later screened for the presence of a mutation(s) via the PCR Rel2F-4R assay. This PCR assay revealed the presence of modified products in 8/11 G_0 flies (Fig. 2B). We observed deletions of several hundred base pairs, but smaller deletions were also detectable. Some flies (flies I, K, and L) presented more than one mutated allele. Interestingly, we could not detect the PCR product corresponding to the wt allele in fly C, suggesting that the CRISPR cleavage might have occurred at a very early stage of fly C development. We then purified and sequenced PCR products corresponding to *rel* mutant alleles (Fig. 2C). As we were hoping to facilitate the screen for mutations in the later generations, we analyzed and maintained only those alleles corresponding to large deletions. With the exception of the PCR product of fly J for which we were unable to obtain a readable sequence, the effective sgRNA(s) could be identified for each analyzed allele, showing at least one effective cutting site for sgRNAs a, b and c but not sgRNA d. Some of the deletions comprised the start codon sequence (flies B, I, and K), whereas others started from exon 2 (flies C, D, E, K, and L). The sequence of the PCR product from fly K was readable only in part, but we identified two different deletions and were able to attribute the effective sgRNAs for both. The mutation in fly L could be deciphered only by looking at later generations and is discussed below (Fig. 3D). We then looked at the predicted protein resulting from each mutant allele (Fig. 2D). Alleles B, I, and K, lacking the start codon sequence as well as a part of the promoter region, should fail to encode any protein. Alleles C, D, E, and L should generate a frameshift mutation, leading to a premature stop codon and production of a truncated protein lacking all critical functional domains. Taken together, these results show that our strategy allowed us to obtain in G_0 several mutant *rel* alleles, predicted in each case to be *null* mutant alleles. While the number of injected individuals reaching adulthood was low (11/540), the proportion carrying one or more mutant alleles was high (8/11), showing high efficiency of the CRISPR/Cas9 editing in *P. papatasi* sand flies.

Germline transmission of mutant alleles. We obtained progeny from all of our 8 G_0 flies showing one or more *rel* mutant alleles. A mutation occurring in G_0 and affecting germ line cells is transmitted to the progeny, and the G_1 individuals can be either wt or a heterozygous mutant. The G_1 females from a given G_0 were sorted at the pupal stage, subjected to mass crossing with wt males, and later pooled as groups of



G_0 fly	cutting site(s)	mutation(s)	resulting protein
A - ♂	X	none	wt
B - ♂	c	364bp deletion	missing start
C - ♀	b	272bp deletion	premature stop
D - ♀	b	338bp deletion	premature stop
E - ♀	b	327bp deletion	premature stop
G - ♀	X	none	wt
H - ♂	X	none	wt
I - ♀	c	364bp deletion	missing start
J - ♂	?	?	?
K - ♀	a and b	<153bp deletion 13bp insertion +320bp deletion	missing start
L - ♀	c	556bp deletion	premature stop

D

wt MSTILDLDSHRVYDTRAFSSTPSPLSNSSTSDGSEATYNYQGSQGSNIGQLSYNMNQMLHLNTAQPHLVIIHEPMDKFRFRYKSEMHGSHGSLMAATKDKSKKHYPRVEL

B ----------
C MSTILDLDSHRVYDTSFVAMMVRPLFDVRYIRKTLNVLCLILTNLSSEMAMKIAKIRMMLSSRQPLASMRNFAAWESYIQPRRILLRSFLRSstop----------
D MSTILDLDSHRVYDTIYSFVAMMVRPLFDVRYIRKTLNVLCLILTNLSSEMAMKIAKIRMMLSSRQPLASMRNFAAWESYIQPRRILLRSFLRSstop----------
E MSTILDLDSHRVYDTRTIEALKIFPPLstop----------
I ----------
K ----------
L MSTILDLDSHRVYDSLAPHLLCPILPPVTEVRQHTMLSFERRPstop----------

FIG 2 Generation of *rel* mutant alleles in G_0 injected flies. (A) Summary of the fate of the embryo injections performed on the same day. (B) Rel2F-4R genotyping-PCR analysis of the 8 G_0 survivors carrying at least one readily detectable mutant *rel* allele(s). (C) Description of identified mutant alleles present in G_0 mosaic individuals. (D) Predicted proteins resulting from the transcription and translation of the identified mutant alleles.

1 to 3, after which they were allowed to lay eggs followed by PCR screening for mutations. We detected one or more heterozygous G_1 females in the progeny of every G_0 mosaic fly, indicating that the mutations generated by CRISPR/Cas9 treatment affected the germ line cells of the G_0 individuals and were transmissible (Fig. 3A). Interestingly, the fly C progeny in G_1 was composed of only 2 males and 1 female, all heterozygous for the mutant allele identified in G_0 parent C, which does not exclude the possibility that fly C was homozygous for this allele. For each subsequent generation, we kept only the crosses showing one or more flies carrying a mutant allele.

Isolation of mutant alleles and establishment of homozygous lines. Given the technical difficulty of maintaining and blood feeding a high number of single-pair crosses, we continued to work with only 3 mutant alleles: rel^B, rel^E, and rel^L. A record of each mutant allele in every generation is presented in Fig. 3B. A homozygous stock was obtained for rel^B at G_5 but was unfortunately lost at G_8. Of note, we observed strong seasonal variations in the overall survival rates of our crosses. The loss of flies in generation G_8 was particularly dramatic, as the entire population of our homozygous

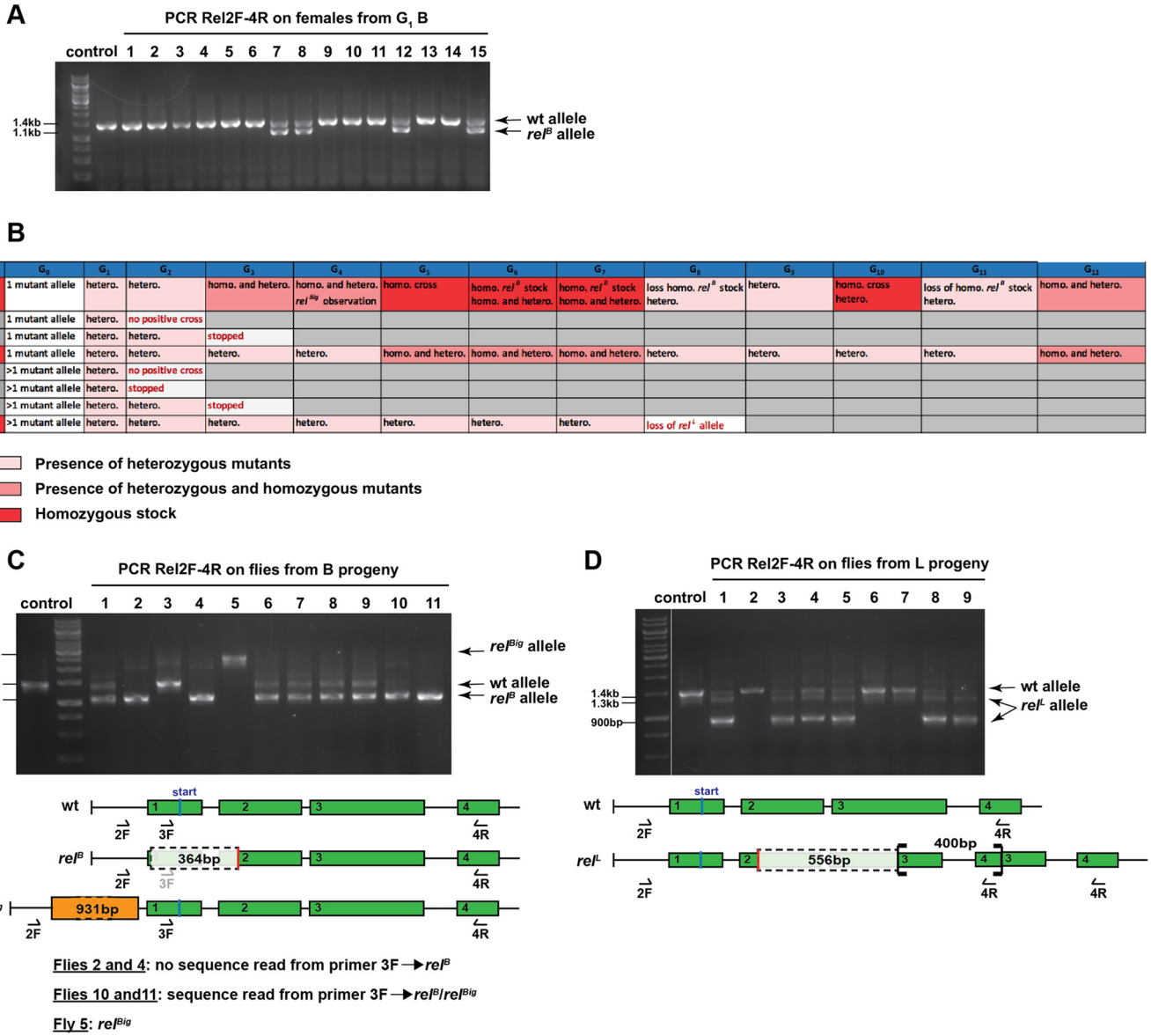


FIG 3 Transmission and isolation of *rel* mutant alleles. (A) Example of mutant allele screening by Rel2F-4R PCR in G_1 females, descended from G_0 B fly. Females 7, 8, 12, and 15 were heterozygous rel^B/wt, whereas the others were wt. (B) Summary of the mutant alleles descended from all mosaic G_0 flies. (C) Identification of rel^{Big} allele in the progeny of fly B. In this example, fly 5 was homozygous for the rel^{Big} allele and flies 2, 4, 10, and 11 were initially recorded as homozygous rel^B. Sequencing with primer 3F later showed that only flies 10 and 11 were indeed homozygous rel^B, while flies 2 and 4 were heterozygous rel^B/rel^{Big}. A schematic representation of the rel^{Big} allele, involving a 931-bp insertion upstream of *rel* exon 1, is shown. (D) Identification of rel^L allele, showing that a 900-bp and a 1.3-kb PCR product always segregated together and were representative of one single allele (flies 1, 3, 4, 5, 8, and 9). Sequencing of the larger product revealed a complex genomic rearrangement, constituting a 556-bp deletion followed by a 400-bp duplication, creating a second hybridization site for primer 4R. The intensity of the molecular wt markers was digitally increased for better visualization.

rel^B stock died, together with all of the flies carrying allele rel^L. Furthermore, no homozygous rel^E or rel^B flies were observed in the genotyped flies surviving from that generation. Seasonal variation is also observed in the wt colonies of flies that we routinely maintain and might reflect changes in the composition of the bacterial communities present in these insects.

Interestingly, in performing genotyping-PCR of fly B progeny, we observed the presence of an unexpected 2.4-kb PCR product from $\geq G_4$ flies. Sequencing identified it as another mutant allele, referred to here as rel^{Big}, corresponding to a 931-bp insertion before the start codon (Fig. 3C; see also Fig. S4). When present, this allele was detected only alone and never in combination with either the wt or the rel^B allele. We

hypothesized that due to its much larger size, the wt or the *rel*^{B} allele might have been preferentially amplified compared to *rel*^{BBig} in *rel*^{BBig}/wt or *rel*^{BBig}/*rel*^{B} heterozygous mutants. This hypothesis was confirmed when we sequenced flies initially identified by PCR as homozygous *rel*^{B} mutants and were able to read a sequence that was absent in the *rel*^{B} allele but present in *rel*^{BBig} (Fig. 3C). From generation G_5, a second PCR was performed in the B family flies in addition to the Rel2F-4R PCR to verify the absence of the *rel*^{BBig} allele, using primers Rel3F (deleted in the allele *rel*^{B}) and Rel4R. Taken together, these data show that a PCR assay, while a convenient screen for mutant alleles, can fail to detect certain mutations such as large insertions and that sequencing may also be necessary to verify the integrity of a mutant stock.

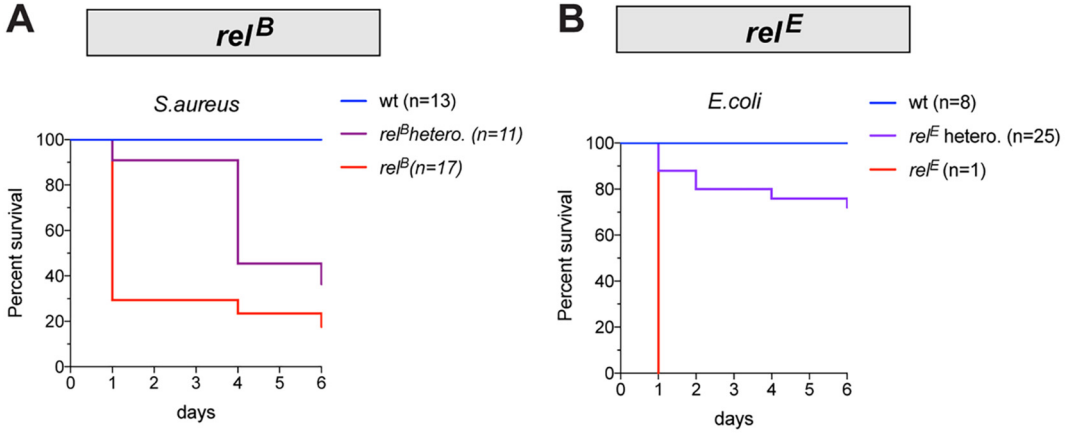
The PCR performed on G_0 fly L generated several PCR products in addition to that corresponding to the wt allele (Fig. 1C). We observed in later generations that a 900-bp product and a 1.3-kb product were never found separately (Fig. 3D). Sequencing of the 1.3-kb product revealed that the two PCR products actually correspond to a single mutant allele, composed of a 556-bp deletion in exons 2 and 3 followed by a 400-bp duplication of part of exons 3 and 4, creating a second hybridization site for the primer Rel4R used in our PCR assay (Fig. 3D). This result indicates that the CRISPR/Cas9 technique can generate genomic rearrangements more complex than simple deletions or insertions in the *P. papatasi* genome.

Together, these data show that several mutant alleles generated by CRISPR/Cas9 were identified and maintained. We successfully established a homozygous stock for allele *rel*^{B} in generation G_5 but lost it three generations later, at the same time as the stock carrying allele *rel*^{L}. We continue to maintain alleles *rel*^{B} and *rel*^{E} by individual and mass crosses.

Relish is an important actor in the sand fly antibacterial response. The role of the IMD pathway in *Drosophila* in defense against Gram-negative bacteria such as *Escherichia coli* and against Gram-positive *Staphylococcus aureus* was described previously (3, 4, 32). We tested the immune response of our *P. papatasi rel* mutants by oral exposure of <7-day-old males to 20% sucrose mixed with either *S. aureus* or *E. coli* followed by counting the number of survivors daily. The males were picked from the progeny of mass crosses or from the homozygous *rel*^{B} stock (G_7). While all ($n = 13$) of the wt males exposed to *S. aureus* survived more than 6 days, 70% (12/17) of *rel*^{B} homozygous mutants died less than 1 day after exposure, and only 83% (14/17) were dead by day 6. *rel*^{B} heterozygous mutants presented an intermediate phenotype, with 64% (7/11) dead within 6 days after *S. aureus* exposure (Fig. 4A). All of the wt males exposed to *E. coli* also survived ($n = 13$), while 28% (7/25) of the heterozygous *rel*^{E}/wt mutants died by day 6 (Fig. 4B). The only exposed *rel*^{E} homozygous mutant died within the first day.

The expected and observed frequencies of wt, heterozygous, and homozygous genotypes in the fertile progeny of the G_2-to-G_8 single-pair crosses containing at least one copy of the *rel*^{E} allele are given in Fig. 4C. The *rel*^{E}/wt $\times$ *rel*^{E}/wt single-pair crosses generated only 2% of fertile *rel*^{E} homozygous adult progeny, whereas 25% were expected by Mendelian proportions. The low proportion of *rel* homozygous mutants indicates a survival defect likely due to high sensitivity to microbial infections, consistent with our oral exposure results. Altogether, these data highlight that the *P. papatasi rel* mutants present a defective antibacterial immune response.

The gut microbiota is modified in *rel* mutant flies. Previous studies reported that the sand fly gut microbiota plays a crucial role in its vector competence for *Leishmania* (33, 34). With this in mind, we compared the relative abundances of bacterial species between small groups of wt and *rel*^{B} heterozygous or homozygous mutants in the absence of *Leishmania* infection. Considerable variation was observed in the composition of the microbiota within each group (Fig. S5 and S6); however, this variability was also detected between different groups of wt flies, consistent with our previous findings (34). In order to compare the quantities of bacteria colonizing the guts of *rel* mutant and wt flies during *Leishmania* infection, we performed 16S quantitative PCR



C

	single-pair crosses performed	single-pair crosses with adult progeny	fertile adult progeny genotype			
				wt	rel^E/wt	rel^E
rel^E/wt x wt	36	11	expected	50%	50%	0%
			observed	34=59%	24=41%	0
rel^E/wt x rel^E/wt	35	18	expected	25%	50%	25%
			observed	35=34%	66=64%	2=2%
rel^E x rel^E/wt	1	1	expected	0%	50%	50%
			observed	0	4=100%	0

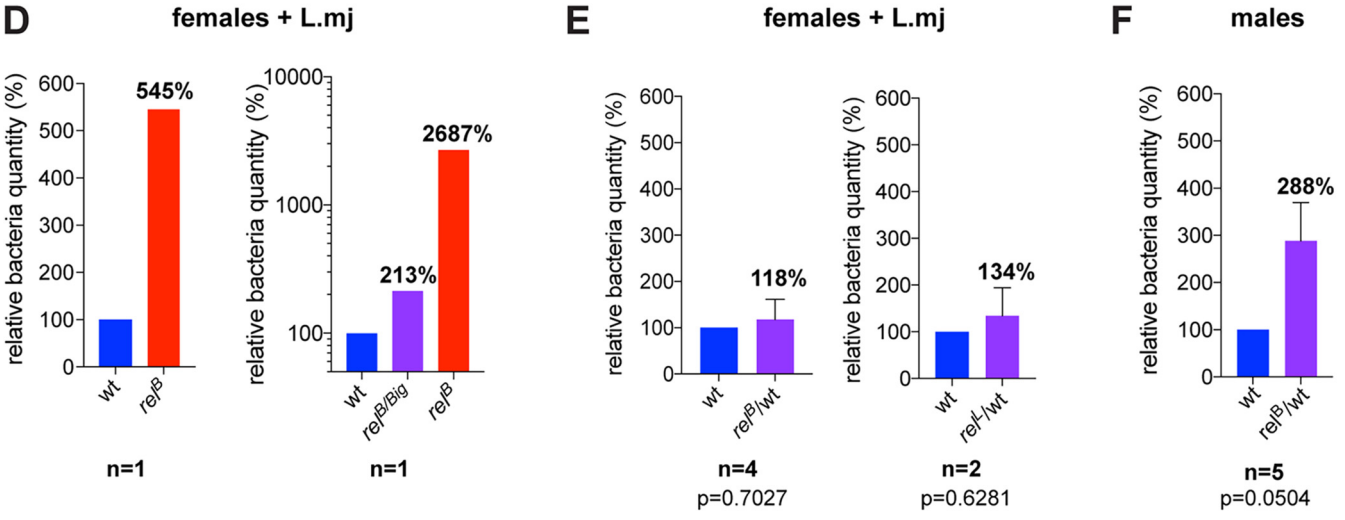


FIG 4 *P. papatasi rel* mutants present a defective antibacterial immune response and increased colonization by gut bacteria. (A) Survival assay of wt and rel^B heterozygous and homozygous sand fly males orally exposed to *S. aureus*. (B) Survival assay of wt and rel^E heterozygous and homozygous sand fly males orally exposed to *E. coli*. (C) Observed and expected numbers of wt, heterozygous, and homozygous rel^E sand flies in the progeny of the G_2 to G_8 single-pair crosses containing at least one copy of the rel^E allele (only the adults producing a progeny were genotyped). (D) Comparison of the relative quantities of bacteria in the guts of wt, $rel^{B/Big}$, and rel^B homozygous mutant females infected with *L. major* strain *Ryan* (L.mj). The two graphs represent results from independent experiments performed with groups of 10 (left) and 4 (right) pooled guts. (E) Comparison of the quantities of bacteria in the guts of wt and rel^B/wt or rel^L/wt heterozygous females infected with *L. major* strain *Ryan* by 16S qPCR. Values shown on the left graph (rel^B/wt) represent means + 1 standard deviation (SD) of results from 4 independent experiments performed with groups of 11 (2 experiments), 10 (1 experiment), or 6 (1 experiment) pooled guts. Values on the right graph (rel^L/wt) represent means + 1 SD of results from 2 independent experiments performed with groups of 9 or 6 pooled guts. (F) Comparison of the quantities of bacteria in the gut of wt and rel^B/wt heterozygous males. Values shown represent means + 1 SD of results from 5 independent experiments performed with groups of 10 pooled guts (4 experiments) or 6 pooled guts (1 experiment).

(qPCR) experiments on pooled guts of females infected with *L. major*. The quantities of 16S DNA observed in homozygous rel^{B} infected females were 5.45 times greater than those of the wt females infected and dissected at the same time (Fig. 4D, left). Due to the loss of the rel^{B} homozygous stock, this particular comparison could not be repeated. However, we also infected the female progeny of mass crosses of flies with rel^{B} and rel^{L} mutant alleles, corresponding to a mix of wt, heterozygous, and homozygous mutants. After dissection, the genotype of these females was identified by PCR using the head as a source of DNA. The genotyping revealed an excess of wt and very few homozygous mutants in each member of the mixed population of flies, highlighting again the fitness costs to flies bearing the *rel* null mutant alleles. Nonetheless, 4 rel^{B} homozygous mutants were found in one experiment, and their pooled gut bacterial quantity was found to be 2,687% of that of the wt flies, with an intermediate value of 213% observed in the heterozygous group (Fig. 4D, right). Several independent experiments performed with larger groups of pooled guts (6 to 11) were combined to compare the relative bacterial loads of wt and heterozygous rel^{B}/wt or rel^{L}/wt infected females, and no significant differences were reached (Fig. 4E). The male progeny of mass crosses for the rel^{B} allele were also analyzed, and the quantity of 16S DNA observed in heterozygous rel^{B}/wt males was 288% of that of wt males (Fig. 4F) (5 independent experiments, $n = 0.0504$). Together, our results show that the gut microbiota could be altered in terms of the quantity and diversity of the bacteria in the *rel* mutant flies.

The IMD pathway controls *P. papatasi* vector competence for *L. major*. To test the influence of the IMD pathway on the ability of *P. papatasi* to carry *L. major* parasites, we infected *rel* homozygous mutants with *L. major* by artificial blood-feeding. We observed a significant increase ($P = 0.0049$) in the number of promastigotes in the midguts of rel^{B} infected females compared to the wt at day 9 after infection (Fig. 5A). Due to the loss of the homozygous rel^{B} stock, we were unable to repeat this experiment or investigate additional time points. We were, however, able to infect the female progeny of mass crosses for the rel^{B}, rel^{E}, and rel^{L} alleles. The genotyped populations were again composed of an excess of wt and heterozygous mutants. The presence of the rel^{Big} allele was also observed in some of the experiments performed with the rel^{B} population. Compared to the wt females, we observed an increased number of *L. major* promastigotes in the guts of the *rel* mutant flies (Fig. 5B to D; heterozygous and rare homozygous mutants, indicated in red, were grouped together). Although a trend toward increased infection intensity was observed for each of the mutant alleles, a significant difference was reached only in the total number of day 7 promastigotes in the rel^{E} mutants (Fig. 5C) and for the increased number of late-stage metacyclic promastigotes in the heterozygous rel^{B} mutants (rel^{B}/wt + $rel^{B/Big}$) (Fig. 5D; right panel). We observed no homozygous mutants in the rel^{L} mass cross population.

DISCUSSION

In this report we present the CRISPR/Cas9 strategy used in *P. papatasi* sand flies to knock out the gene encoding the IMD-pathway transcription factor Rel. By injecting four different sgRNAs targeting the first and second exons of *rel* together with a recombinant Cas9 protein, we generated several *rel null* mutant alleles that were easily detectable by PCR screening and transmissible from generation to generation. Although rare and ultimately lost as an inbred line due to their decreased fitness, *rel* homozygous null mutants were able to be used in a few experiments. The *null* mutants presented a higher susceptibility to bacterial challenge and a higher quantity of bacteria in their guts than the wt flies and were more permissive to *L. major* growth and development. Following loss of the *null* mutant stock, we relied on flies carrying different heterozygous mutant alleles to try to substantiate a role for *rel* in vector competence. Of the three lines carrying the different heterozygous rel^{-}/wt mutant alleles, two presented significantly greater numbers of parasites per gut or greater numbers of metacyclic promastigotes. Note that the wt *P. papatasi* stock has in our hands shown a relatively poorly permissive phenotype for *L. major* growth and devel-

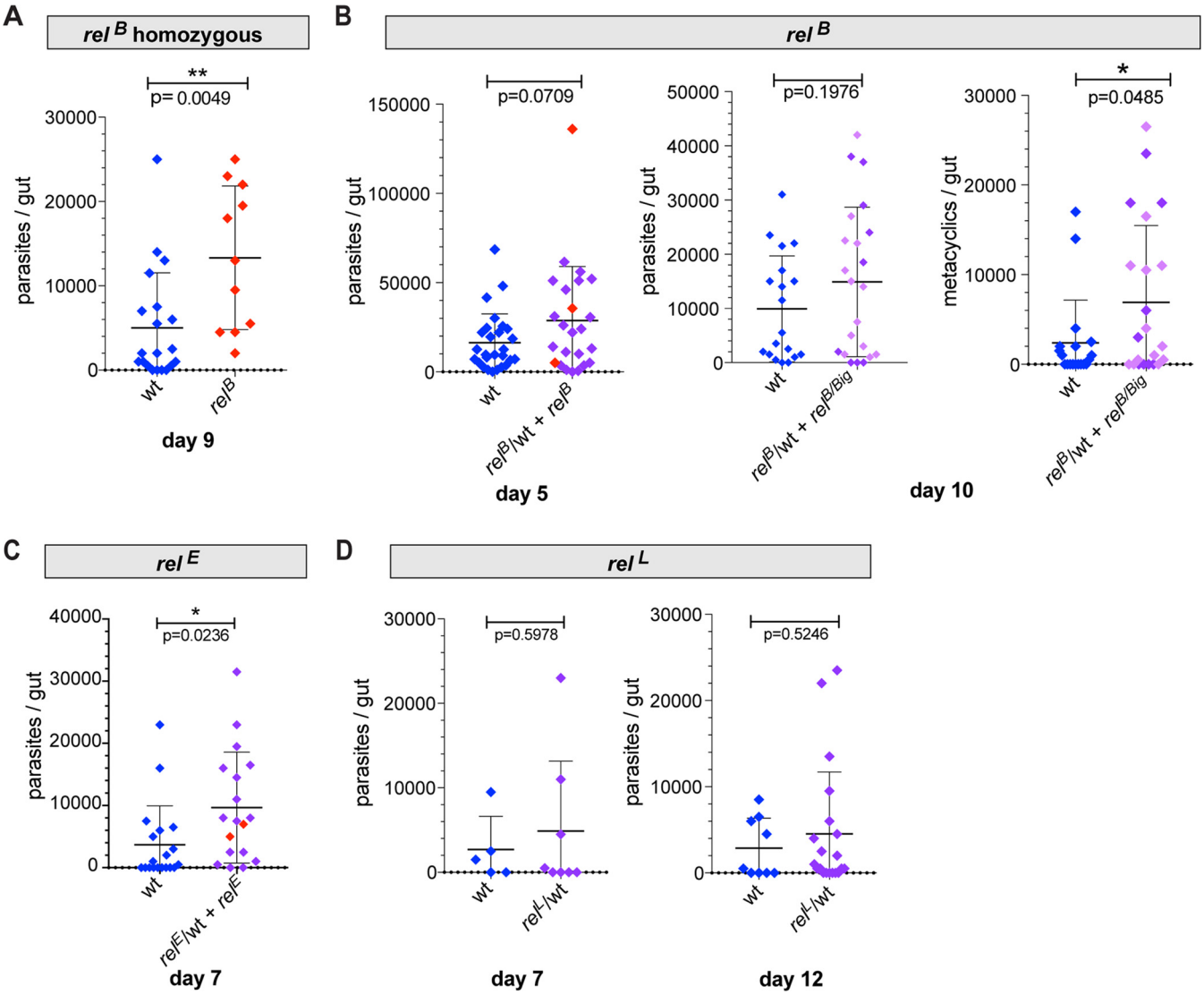


FIG 5 *P. papatasi rel* mutants show increased permissiveness to *L. major*. (A) Total numbers of *L. major* strain *Ryan* promastigotes in the gut of wt and rel^B mutant females infected with *L. major* strain *Ryan*. (B) Total numbers of *L. major* strain *Ryan* promastigotes in the gut of infected females descended from mass crosses of flies carrying the rel^B allele. Heterozygous (rel^B/wt or $rel^{B/Big}$) individuals are represented in purple; homozygous mutants are indicated in red. (C) Total numbers of *L. major* strain *Ryan* promastigotes in the gut of infected females descended from mass crosses of flies carrying the rel^E allele (homozygous and heterozygous mutants grouped together; homozygous mutants are designated in red). (D) Total numbers of *L. major* strain *Ryan* promastigotes in the gut of infected females descended from mass crosses containing the rel^L allele (no homozygous rel^L mutants were present in these experiments). The day of dissection postinfection is indicated below each graph. Values shown represent parasite numbers per individual midgut, with means ± 1 standard deviation.

opment. The current results indicate that the sand fly immune response, through Rel and, more generally, the IMD pathway, can negatively impact sand fly vector competence for *Leishmania*.

So far as we are aware, this work represents the first successful instance of CRISPR/Cas9 technology being applied to sand flies. While the proportion of the injected embryos surviving to adults was low (11/540), the efficiency of mutagenesis was high (8/11). The mutant alleles, which included in most cases several hundred base pair deletions, were transmitted from generation to generation, showing that the Cas9 nuclease DNA break(s) affected the germ line. A homozygous mutant stock could be established for a few generations but was then lost, likely due to the high sensitivity of *rel* mutant flies to microbial colonization.

In our analysis of the mutant alleles present in the G_0 adults, the *rel* wt allele was not detected by our screening-PCR for one of the injected individuals (fly C; Fig. 2B). As a

consequence of injection of the sand fly eggs at a very early step in embryo development (within 4 h after they were laid), the absence of the wt allele in fly C could reflect a very early transformation event, leading to a homozygous mutation instead of a mosaic genotype. Compatible with this hypothesis, after being crossed with wt males, fly C died soon after producing only 3 progeny flies, all carrying the mutant allele detected in G_0.

Using the PCR rel2-4 assay for genotyping the flies, we were surprised to observe the presence of unexpected PCR products, such as the one encoding the *rel*^{Big} allele, after a few generations (Fig. 3C). This allele could be detected only in the homozygous state, revealing some bias in the PCR strategy that we used to genotype the flies. In contrast to all of the other *rel* mutant alleles identified, no cleavage site predicted from the sgRNAs could be identified for the *rel*^{Big} allele. The 931-bp insertion present in *rel*^{Big} was subjected to BLAST analysis in the (imperfect) genomic sequence available for *P. papatasi* and aligned with an unidentified genomic region (see Fig. S4 in the supplemental material). We do not know when the genome modification leading to the presence of the *rel*^{Big} allele occurred, but it is possible that it happened as early as G_0, was maintained from generation to generation in our *rel*^{B} population, and was detected only when present in the homozygous state.

Another allele of particular interest is *rel*^{L}, consisting of a deletion in exons 2 and 3 followed by a duplication of parts of exons 3 and 4 (Fig. 3D). This allele illustrates that genomic rearrangements more complex than insertion or deletion can occur and raises the issue of the nature of the genetic mechanism used in the sand fly cell nuclei to repair the DNA break(s) mediated by the Cas9 nuclease in G_0. As we did not include a donor template to repair the DNA using HDR, we were expecting the results to show only deletions or insertions generated by NHEJ, thought to be the preferred DNA repair pathway in insects. In contrast, it is possible that the *rel*^{L} allele was obtained an a consequence of the activity of a HDR pathway, perhaps using the wt allele on the homologous chromosome as a DNA template to repair a DNA break occurring on the first. This could indicate that more-complex CRISPR-Cas9 strategies, using donor templates for inserting a sequence of interest into a targeted region in the genome, are possible in sand flies.

Our observations indicating that Rel and, more generally, the IMD pathway limit the quantity of bacteria present in the sand fly gut, as well as the permissiveness of these flies to *Leishmania*, are consistent with a number of published findings. RNA interference targeting Caspar previously showed that this negative regulator of the IMD pathway positively impacts *L. mexicana* numbers or *L. infantum* numbers in the gut of *Lutzomyia longipalpis* sand flies (14). Given the crucial role that gut microbial communities have been shown to play in the vector competence for *Leishmania* (33, 34), and our observation that the composition of the gut microbiota in *rel* mutant flies is altered, it is possible that the role of the fly immune response in the control of *Leishmania* growth and development was secondary to the changes in microbiota size and diversity rather than representing a direct effect on the parasites themselves.

Finally, while our CRISPR strategy focused only the IMD pathway, other pathways are of crucial importance in the insect immune response (3, 4). A role for the Toll pathway seems especially likely in the sand fly response to *Leishmania* because, as with the IMD pathway, its activation was observed *in vitro* when cultured sand fly cells were exposed to bacteria or *Leishmania* (11), and upregulation of a gene encoding a defensin AMP, whose expression in *Drosophila* depends on Toll (3), was observed in flies infected with *L. major* (12, 13).

To conclude, by targeting the transcription factor of the key immune response IMD pathway, we present the first example of successful *in vivo* sand fly mutagenesis by CRISPR/Cas9 and substantiate an important role of this pathway in the control of sand fly vector competence for *Leishmania*. The demonstration that CRISPR/Cas9 genome editing can be adapted to sand flies opens the possibility of investigating other intrinsic sand fly factors that are believed to influence the development of transmissible infections, such as midgut proteases and parasite attachment sites (35).

MATERIALS AND METHODS

Ethics statement. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). The protocol was approved by the Animal Care and Use Committee of the NIAID, NIH (protocol number LPD 68E). Invertebrates are not covered under NIH guidelines.

CRISPR/Cas9 injection mix. The CRISPR/Cas9 injection mixture was prepared as described previously (24), using 300 ng/μl Cas9 recombinant protein (PNABio) together with 80 ng/μl sgRNAs a, b, c and d. The sgRNAs were synthetized by *in vitro* transcription on PCR templates by the use of a MEGAscript *in vitro* transcription kit (Ambion) and were purified using a MEGAclear kit (Invitrogen). The sequences of the primers used for generating the PCR templates and the PCR conditions as well as the sequences of the sgRNAs are given in Fig. S2 in the supplemental material. The embryo injections were performed at the Insect Transformation Facility of The University of Maryland. Females were collected and allowed to lay eggs 5 days after blood feeding by being transferred from a dry to a moist pot. The resulting early embryos were injected a maximum of 4 h after the eggs were laid. Detailed protocols for sand fly egg collection and injection are provided in Fig. S7.

Sand fly rearing and maintenance of mutant alleles. After injection, the embryos were transferred to moist plaster pots on the same day. Drops of 0.5% propionic acid were added to the pots 24 h later to prevent fungal contamination. Dead embryos were removed every day, and hatching larvae were carefully transferred into new pots. For every generation, pupae were separated by sex to maintain the females as virgins until they were used in designated crosses. After the selected sand flies were crossed, the females were blood-fed on anesthetized mice. Adults to be genotyped were collected after eggs were obtained and kept at −20°C until being genotyped with a Phire-PCR kit (Thermo Fisher), following the manufacturer's protocol, with primers Rel2F and Rel4R. Some PCR products were also purified (gel purification or total purification performed with a QIAquick gel extraction kit [Qiagen] or a QIAquick PCR purification kit [Qiagen], respectively) and later sequenced with an Rel2F or Rel3F and/or Rel4R primer to identify or to confirm the identity of the mutant alleles. At generation G_5 in the flies descending from the G_0 B adult, another PCR was performed in addition to the Rel2F-4R PCR to check for the presence of the *rel*^{Big} allele, using primers Rel3F (deleted in the *rel*^{B} allele) and Rel4R. The sequence of each of the primers is given in Fig. S3.

Bacterial feeding and survival assays. The following stocks of bacteria were used: *E. coli* K-12 and *S. aureus* SH1000. Sand fly males were starved overnight before being exposed to bacteria. Bacteria were cultured overnight in LB liquid medium at 37°C and diluted 1/50 the next morning. After a few hours, the optical density at 600 nm (OD_{600}) was measured and the bacterial culture was diluted in a solution of 20% sucrose in order to obtain approximately 10^7cells/ml, based on the Agilent *E. coli* OD calculator. The solution was mixed with a 1/30 dilution of green alimentary dye (Spice Supreme food colors; Amazon) for detection of fed flies, and the flies were exposed to the resulting solution until the next morning. For survival assays, the dead flies were counted and collected each day. PCR genotyping was performed on the dead bodies as described in the previous section.

Gut microbiota analysis. The isolation and identification of the gut bacteria in the wt or *rel* mutant *P. papatasi* flies, as well as the determination of their relative abundance levels, were performed as previously described (34). Comparisons of the gut bacterial loads of wt and mutant flies were performed by 16S qPCR as follows. Flies were dissected, and guts and heads were collected individually in 50 μl and 5 μl of phosphate-buffered saline (PBS), respectively, and were then kept at −20°C. The heads were used as a source of DNA for the genotyping, performed using PCR Rel2-4 assay and a Phire-PCR kit. After genotyping, gut homogenates from flies of same genotype were pooled in order to form groups with the same initial number of guts. DNA was extracted from these samples using a DNeasy blood and tissue kit (Qiagen). Quantitative PCRs were performed on these DNA extracts by the use of the 16S primers CS1 and CS2, which are specific to bacterial DNA, and primer Lls6, recognizing a sand fly housekeeping gene (primer sequences are given in Fig. S3). The relative quantities of bacteria per gut were calculated using the threshold cycle ($\Delta\Delta C_T$) method.

Sand fly infections with *Leishmania* parasites. Maintenance of the *P. papatasi* sand flies and their infection with cultured *L. major* strain *Ryan* were performed as previously described (34). Briefly, 2-to-4-day-old female sand flies were infected by artificial feeding through a chick-skin membrane containing heparinized, heat-inactivated mouse blood and 4×10^6/ml logarithmic-phase promastigotes. At different times postinfection, midgut homogenates were prepared and deposited on a hemocytometer to count the total numbers of promastigotes as well as the numbers of metacyclic promastigotes per midgut. For infections of the progeny of mass crosses, the heads of the corresponding flies were saved in an Eppendorf tube containing 5 μl of PBS and were then used as a source of DNA for genotyping. The genotyping was performed as described above.

Statistical analyses. Student's *t* test was used to assess significant differences in bacterial and parasite counts between wild-type and *rel* mutant flies.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.01941-19.

FIG S1, DOCX file, 0.02 MB.

FIG S2, DOCX file, 0.1 MB.

FIG S3, DOCX file, 0.01 MB.

FIG S4, DOCX file, 0.02 MB.
FIG S5, TIF file, 2 MB.
FIG S6, TIF file, 1.8 MB.
FIG S7, DOCX file, 0.1 MB.

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We declare that we have no conflicts of interests.

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