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Gene knock out of honey bee trypanosomatid parasite, *Lotmaria passim*, by CRISPR/Cas9 system

Qiushi Liu and Tatsuhiko Kadowaki

Department of Biological Sciences, Xi'an Jiaotong-Liverpool University, 111 Ren'ai Road, Suzhou Dushu Lake Higher Education Town, Jiangsu Province 215123, China

Corresponding author:
Tatsuhiko Kadowaki
Department of Biological Sciences, Xi'an Jiaotong-Liverpool University
111 Ren'ai Road, Suzhou Dushu Lake Higher Education Town
Jiangsu Province 215123, China
TEL: 86 512 88161659, FAX: 86 512 88161899
E-mail: Tatsuhiko.Kadowaki@xjtlu.edu.cn

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19 **Abstract**

20 Two trypanosomatid species, *Lotmaria passim* and *Crithidia mellificae,* have been
21 shown to parasitize honey bees to date. *L. passim* appears to be more prevalent than *C.*
22 *mellificae* and specifically infects the honey bee hindgut. Although the genomic DNA
23 has been sequenced, the effects of infection on honey bee health and colony are poorly
24 understood. To identify the genes that are important for infecting honey bees and to
25 understand their functions, we applied the CRISPR/Cas9 system to establish a method
26 to manipulate *L. passim* genes. By electroporation of plasmid DNA and subsequent
27 selection by antibiotics, we first established an *L. passim* clone expressing tdTomato,
28 GFP, or Cas9. We also successfully knocked out the endogenous miltefosine transporter
29 and tyrosine amino transferase genes by replacement with antibiotics (hygromycin)
30 resistant gene using the CRISPR/Cas9-induced homology-directed repair pathway. The
31 *L. passim* clones expressing fluorescent markers, as well as the simple method for
32 knocking out specific genes, could become useful approaches to understand the
33 underlying mechanisms of honey bee-trypanosomatid parasite interactions.

34

35 **Key words**: Honey bee, Trypanosomatid, *Lotmaria Passim*, CRISPR/Cas9, Genome
36 editing

37

38

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39 **Introduction**

40 The honey bee (*Apis mellifera*) plays important roles in agricultural crop production and
41 ecosystem conservation across the globe (Klein et al., 2007;Aizen et al., 2008;Potts et
42 al., 2010). However, a decline in managed honey bee colonies has been observed in
43 North America, Europe, and a part of Asia since 2006. The underlying reasons for the
44 large-scale colony losses are complex, but can be divided into several different
45 categories: inadequate food supplies, anthropogenic chemicals, and exposure to various
46 pathogens/parasites (Goulson et al., 2015). There are diverse honey bee
47 pathogens/parasites, such as viruses, bacteria, fungi, protozoans, and mites (Evans and
48 Schwarz, 2011). Among the protozoans, two *Trypanosomatidae, Lotmaria passim* and
49 *Crithidia mellificae*, were shown to infect honey bees. *C. mellificae* was first identified
50 in Australia in 1967 (Langridge and McGhee, 1967). Later, in 2015, another novel
51 trypanosomatid parasite infecting honey bees was discovered and named *L. passim*
52 (Schwarz et al., 2015). *L. passim* was found to be more prevalent than *C. mellificae*
53 (Schmid‐Hempel and Tognazzo, 2010; Morimoto et al., 2013; Cepero et al., 2014;
54 Ravoet et al., 2014; Cersini et al., 2015; Ravoet et al., 2015; Schwarz et al., 2015;
55 Arismendi et al., 2016; Cavigli et al., 2016; Stevanovic et al., 2016; Vavilova et al.,
56 2017; Regan et al., 2018) and fewer honey bee colonies were reported to be infected by
57 *C. mellificae* (Ravoet et al., 2015). Thus, *L. passim* rather than *C. mellificae* is likely to
58 be associated with the previously reported winter mortality of honey bee colonies
59 (Ravoet et al., 2013). However, the effects of *L. passim* infection on honey bee health
60 and colonies is poorly understood. *L. passim* specifically infects the honey bee hindgut
61 and triggers the expression of antimicrobial peptide (AMP) genes, such as *Defensin 1*
62 and *Abaecin*. In addition, *L. passim* stimulates the increased expression of genes
63 encoding several downstream components of immune pathways (honey bee orthologs of
64 Imd and Dscam) (Schwarz and Evans, 2013). Although the genome of *L. passim* has
65 been sequenced (Runckel et al., 2014), both *C. mellificae* and *L. passim* have not been
66 fully investigated to date.

67 *Crithidia bombi*, a trypanosomatid parasite of the bumble bee, has been well
68 characterized. *C. bombi* infection dramatically reduces colony-founding success, male
69 production, and colony size (Brown et al., 2003). Furthermore, *C. bombi* infection was
70 also reported to impair the ability of bumble bees to utilize floral information (Gegear et
71 al., 2006). *C. bombi* infection induces expression of several immune-related genes:
72 *MyD88*, *Relish*, *Thioester-containing protein 7* (Schlüns et al., 2010), as well as AMP
73 such as *Abaecin*, *Defensin* and *Hymenoptaecin* in bumble bees (Riddell et al., 2011;
74 Riddell et al., 2014). The genomes of *C. bombi* and *Crithidia expoeki* were recently

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75 sequenced and the sequences revealed signs of concerted evolution of genes potentially
76 important for interaction with the host (Schmid-Hempel et al., 2018).
77 Recently, a new method based on the CRISPR/Cas9 system has become widely
78 used for genome editing. It has also been applied to edit the genomes of various
79 trypanosomatid parasites: *Trypanosoma cruzi* (Lander et al., 2015a; Peng et al., 2015;
80 Lander et al., 2016; Lander et al., 2017), *Trypanosoma brucei* (Beneke et al., 2017; Rico
81 et al., 2018), *Leishmania major* (Sollelis et al., 2015; Beneke et al., 2017), *Leishmania*
82 *donovani* (Zhang and Matlashewski, 2015; Martel et al., 2017; Zhang et al., 2017), and
83 *Leishmania mexicana* (Beneke et al., 2017). Although the nonhomologous end-joining
84 (NHEJ) pathway appears to be absent in trypanosomatid parasites (Passos-Silva et al.,
85 2010), the endogenous genes were successfully knocked out both by the
86 microhomology-mediated end joining (MMEJ) and homology-directed repair (HDR)
87 pathways, in order to repair Cas9-induced double-strand DNA breaks (DSBs). In this
88 study, we first generated *L. passim* clones expressing fluorescent markers and then
89 attempted to use CRISPR/Cas9 for genome editing. We will discuss how these
90 approaches can be used to better understand honey bee-trypanosomatid parasite
91 interactions.
92

93 **Materials and Methods**

94 **Culture of *L. passim***

95 *L. passim* strain SF (PRA-403) was obtained from the American Type Culture
96 Collection (ATCC) and cultured in the modified FPFB medium (Salathe et al., 2012).
97 The parasite lines with fluorescent markers were cultured in the modified FPFB
98 medium containing 5 μg/mL blasticidin (InvivoGen). The gene knocked-out parasite
99 lines were maintained in the presence of 5 μg/mL blasticidin, 10 μg/mL hygromycin
100 (SIGMA), 50 μg/mL G418 (SIGMA). To monitor the growth rate, *L. passim* was first
101 inoculated at 5 x 10^5/mL, and then the number of parasites during the culture was
102 measured by CASY® Cell Counter together with Analyzer System Model TT (OMNI
103 Life Science).

104 **Electroporation of *L. passim* followed by the single clone selection**

105 Actively growing *L. passim* was collected, washed twice, and resuspended in 0.4 mL of
106 Cytomix buffer (20 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 25 mM HEPES and 5
107 mM $MgCl_2$, pH 7.6). 2 x 10^7 of *L. passim* were electroporated with 10 μg of plasmid
108 DNA: pTrex-Neo-tdTomato (Canavaci et al., 2010), pTrex-n-eGFP (Peng et al., 2015),
109 or pTrex-b-NLS-hSpCas9 (Peng et al., 2015) using a Gene Pulser X cell electroporator
110 (Bio-Rad). For co-transfection of sgRNA expression vector and donor DNA, 10 μg of

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111 the plasmid DNA and 25 μg of linearized donor DNA were electroporated. The 2 mm
112 gap cuvettes were chilled on ice for 15 min before electroporation. The voltage,
113 capacitance, and resistance were set at 1500 V, 25 μF, and Infinity, respectively for all
114 experiments. The electroporation was repeated twice and there was 10 sec interval
115 between each pulse. For single clone selection, the electroporated parasites growing in
116 the medium with appropriate antibiotics were diluted and spread on 2.5 % agarose
117 containing the modified FPFB medium and the antibiotics at 25 °C. The individual
118 parasite colonies were picked and expanded in 12-well plate. We used serial dilution for
119 the single clone selection to compare gene knock-out by CRISPR/Cas9-induced HDR
120 and homologous recombination.

121 **Western blot analysis**

122 The parasites expressing Cas9 were directly suspended with the sample buffer for
123 SDS-PAGE and heated for 3.5 min. The cell lysates were separated by two 8 %
124 SDS-PAGE gels and then the proteins in one gel were transferred to a PVDF membrane.
125 Another gel was stained by coomassie brilliant blue as the loading control. The
126 membrane was blocked with 5 % BSA/TBST and then incubated with rabbit anti-FLAG
127 antibody (Sigma-Aldrich, 100-fold dilution) at 4 °C overnight. After washing the
128 membrane three times with TBST, it was incubated with IRDye® 680RD anti-rabbit
129 secondary antibody (10, 000-fold dilution) in 5 % skim milk/TBST at room temperature
130 for 1 h. The membrane was washed as above and scanned/analyzed by Odyssey®
131 scanner (LI-COR Biosciences).

132 **Gene knock-out by CRISPR/Cas9-induced HDR**

133 *L. passim* miltefosine transporter (LpMT) and tyrosine amino transferase (LpTAT)
134 sgRNA sequences were designed using a custom sgRNA design tool
135 (http://grna.ctegd.uga.edu) (Peng et al., 2015). These two sgRNA sequences were
136 cloned into pSPneogRNAH vector (Zhang and Matlashewski, 2015). The donor DNA
137 for *LpTAT* gene was constructed by fusion PCR of three DNA fragments, 5' (438 bp)
138 and 3' UTRs (500 bp) of *LpTAT* and the ORF of hygromycin B phosphotransferase
139 gene derived from pCsV1300 (Park et al., 2013). Similarly, the donor DNA for *LpMT*
140 was prepared as above except the 5' UTR (540 bp) and the part of ORF downstream of
141 the sgRNA target site (500 bp) were used for the fusion PCR. The fusion PCR products
142 were cloned into EcoRV site of pBluescript II SK(+) and the linearized plasmid DNA
143 by HindIII was used for electroporation as mentioned above. After co-transfection, the
144 antibiotics resistant clones were selected as above.

145 **Genomic PCR**

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146 Genomic DNA was extracted from the parasites using DNAiso (TAKARA) and PCR
147 was carried out using KOD FX polymerase (TOYOBO) and the specific primers shown
148 in the Figures and Supplementary Table 1. Some of the PCR products were gel purified
149 and directly sequenced.

150 **Detection of *LpMT* and *LpTAT* mRNAs by RT-PCR**

151 Total RNA was extracted from wild type, *LpMT* and *LpTAT* heterozygous and
152 homozygous KO parasites using TRIzol reagent (SIGMA) and treated by 1U of
153 RNase-free DNase (Promega) at 37 °C for 30 min. 0.2 μg of total RNA was reverse
154 transcribed by ReverTra Ace (TOYOBO) and random primer followed by PCR with
155 KOD FX polymerase (TOYOBO) and gene specific primers listed in Supplementary
156 Table 1.
157

158 **Results**

159 **Generation of *L. passim* expressing fluorescent marker or Cas9**

160 To test if we could generate an *L. passim* clone stably expressing an exogenous protein,
161 *L. passim* was electroporated with plasmid DNA carrying tdTomato and the neomycin
162 resistance gene (*Neo*) driven by the *Trypanasoma cruzi* rRNA promoter
163 (pTrex-Neo-tdTomato) (Canavaci et al., 2010), followed by G418 selection. Several
164 G418-resistant clones were isolated from an agar plate and expanded. As shown in Fig.
165 1, all the parasite cells expressed tdTomato; however, most of them lost the expression
166 after 14 weeks in culture (25 passages) without G418. These results demonstrate that
167 the electroporated plasmid DNA existed as episomal DNA, without integrating into the
168 parasite's chromosomal DNA. The efficiency of transient transfection was low (up to
169 1.21 %), indicating that the selection of stable transfectants by antibiotics is essential.
170 We then introduced plasmid DNA containing Cas9 and the blasticidin resistance gene
171 (*Bsd*) driven by the *T. cruzi* rRNA promoter (pTrex-b-NLS-hSpCas9) (Peng et al.,
172 2015) into *L. passim,* followed by blasticidin selection. Expression of the FLAG-tagged
173 Cas9 protein was confirmed by western blotting (Fig. 2A) and the growth rate of the
174 Cas9-expressing clone in the presence of blasticidin was comparable to that of the wild
175 type without blasticidin (Fig. 2B).

176 **Knockout of miltefosine transporter and tyrosine aminotransferase genes in *L.***
177 ***passim* by Cas9-induced HDR**

178 We first targeted the *L. passim* miltefosine transporter (*LpMT*) gene for knockout by
179 CRISPR/Cas9-induced HDR since it is not an essential gene, and has been successfully
180 knocked out in *Leishmania donovani* previously (Zhang and Matlashewski, 2015;
181 Zhang et al., 2017). We transfected Cas9-expressing *L. passim* with plasmid DNA that

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182 drives the expression of *LpMT*-specific sgRNA and *Neo* under the *L. donovani* rRNA
183 promoter (Zhang and Matlashewski, 2015), and a donor DNA. The donor DNA
184 contained the hygromycin resistance gene (*Hyg*) flanked by 5'UTR (left arm) and a part
185 of ORF downstream of the sgRNA targeting site of *LpMT* (right arm). After the
186 transfection, we selected and expanded the blasticidin-, G418-, and
187 hygromycin-resistant clones. As shown in Fig. 3A, all the antibiotic resistant clones had
188 the *LpMT* knockout allele mediated by *Hyg* insertion through homologous
189 recombination; however, three out of 11 clones also contained the wild type allele,
190 suggesting that they were heterozygous. To confirm the knockout of *LpMT*, we
191 examined the mRNA expression in wild type, heterozygous, and homozygous knockout
192 parasites by RT-PCR. *LpMT* mRNA was absent in the homozygous knockout strain as
193 shown in Fig. 3B. We also successfully knocked out tyrosine aminotransferase (*LpTAT*)
194 using Cas9-induced HDR as described above (Fig. 4). *LpTAT* is one of the *L. passim*
195 genes that becomes upregulated upon infection of the honey bee hindgut. As shown in
196 Fig. 5, the growth rate of *LpMT* or *LpTAT* knockout parasites in culture medium was
197 lower than that of wild type, suggesting that these two genes are not essential for the
198 parasites' survival, but are necessary to support the optimal growth of *L. passim*.
199 To confirm that replacing the endogenous gene with the donor DNA containing
200 *Hyg* was mediated by DNA break-induced HDR rather than homologous recombination,
201 we repeated the knocking out of *LpMT* as described above, together with transfecting
202 wild type *L. passim* with the donor DNA only. After incubation in the antibiotics-
203 containing culture medium for 61 days, ten individual antibiotics-resistant clones were
204 isolated, expanded, and analyzed by genomic PCR. Fig. 6 shows that all of the ten
205 clones subjected to CRISPR/Cas9-induced HDR were homozygous knockouts; however,
206 all of the ten clones subjected to the homologous recombination retained the wild type
207 *LpMT* allele, demonstrating that they were heterozygous knockouts. Furthermore, the
208 803 bp PCR amplicon was absent in three of the clones (#1, 4, and 5) subjected to the
209 homologous recombination, suggesting that the donor DNA was integrated into *LpMT*
210 with an unexpected orientation or into another locus in the *L. passim* genome. Thus,
211 CRISPR/Cas9–induced HDR is able to replace two alleles of an endogenous gene in *L.*
212 *passim* with a single donor DNA containing an antibiotics-resistant gene.
213
214 **Discussion**

215 **Stable expression of exogenous protein in *L. passim***

216 We successfully introduced several plasmids to express exogenous proteins (tdTomato,
217 GFP, and Cas9) in *L. passim*. Although we evaluated the electroporation under various

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218 conditions, the efficiency of transient transfection was quite low (< 1.21 %), so that
219 stable transfectants had to be selected by antibiotics. Although the introduced plasmid
220 DNA does not appear to integrate into the *L. passim* genomic DNA, it is likely to be
221 present in multicopy, since the parasite has to undergo many cell divisions to lose the
222 plasmid DNA. Consistent with the phylogenetic similarity of *L. passim* with other
223 trypanosomatids (Schwarz et al., 2015), the *T. cruzi* and *L. donovani* rRNA promoters
224 are functional for protein and sgRNA expression in *L. passim*. Therefore, various
225 expression plasmids constructed for genome editing of *Trypanosoma* and *Leishmania*
226 by CRISPR/Cas9 could be directly applied to *L. passim* as well. *L. passim* expressing a
227 fluorescent protein, such as GFP or tdTomato, could be useful to monitor how the
228 parasite establishes the infection in the honey bee hindgut.

229 **Gene knockout in *L. passim* by CRISPR/Cas9-induced HDR**

230 We were able to successfully knock out two endogenous genes of *L. passim* by
231 CRISPR/Cas9-induced HDR. In contrast to knocking out a specific gene by
232 homologous recombination, single transfection with sgRNA-expressing plasmid DNA
233 and donor DNA containing an antibiotics-resistant gene was sufficient. However, it
234 usually takes more than 60 days to obtain the homozygous knockout parasite by
235 selecting the antibiotic resistant clones in the culture medium followed by isolating
236 single clones either from agar plates or by serial dilution. Considering that we obtained
237 both heterozygous and homozygous knockouts for *LpMT* and *LpTAT* (Figs. 3 and 4), as
238 well as the low transfection efficiency of *L. passim*, the replacement with the donor
239 DNA probably occurs initially with one allele. CRISPR/Cas9-induced HDR then
240 follows with the second allele, where the first replaced allele serves as the template.
241 This mechanism is similar to the “mutagenic chain reaction” used for converting
242 heterozygous to homozygous mutations in fruit flies (Gantz and Bier, 2015). To shorten
243 the selection period, electroporation of heterozygous knockout parasites with donor
244 DNA containing different antibiotics-resistance genes followed by selection using two
245 antibiotics should be considered. However, if the target gene is essential for the survival
246 of *L. passim*, only heterozygous knockout clones will be selected. This was indeed the
247 case for the paraflagellar rod component par4 (*LpPFR4*) gene, although the *PFR1* and
248 *PFR2* genes have been successfully knocked out in *T. cruzi* (Lander et al., 2015b).

249 We did not observe any alterations in the target gene when we expressed only
250 Cas9 and sgRNA in *L. passim.* After selecting *L. passim* expressing Cas9 and sgRNA
251 using blasticidin and neomycin in the culture medium, the genomic DNA extracted
252 from the pooled parasites or the expanded individual clones was analyzed by
253 sequencing the PCR products encompassing the sgRNA-target site. We did not find any

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254 changes within the DNA sequences, and the same results were also obtained by
255 digesting the PCR products with T7 endonuclease I. As previously reported
256 (Passos-Silva et al., 2010), the NHEJ pathway is absent in trypanosomatids; however,
257 the MMEJ pathway is apparently present in *L. donovani* and *T. cruzi* based on the
258 successful gene modifications by expressing Cas9 and sgRNA (Peng et al., 2015; Zhang
259 and Matlashewski, 2015). In *L. passim*, both the NHEJ and MMEJ pathways might not
260 exist, perhaps because the parasite lacks the essential genes. Alternatively, Cas9
261 introduces only a single-strand break, but not DSBs in *L. passim* genomic DNA, so that
262 only the HDR pathway is induced as a result.

263 We could apply the CRISPR/Cas9-induced HDR to prepare a library of *L.*
264 *passim* clones in which specific genes are knocked out. The genes essential for survival
265 could be identified by the absence of homozygous knockouts, and the genes necessary
266 for optimal growth in the culture medium could also be tested. More importantly, we
267 could infect honey bees with the abovementioned *L. passim* clones, and identify the
268 genes important for establishing and maintaining the infection in the honey bee gut.
269 Understanding the gene functions will provide insights into the molecular and cellular
270 mechanisms of host (honey bee)-parasite (*L. passim*) interactions.

271

272 **Conflict of Interest Statement**

273 The authors declare no conflict of interest.

274

275 **Author contribution**

276 QL conducted all experiments. TK supervised the research project.

277

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288

289 **References**

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290 Aizen, M.A., Garibaldi, L.A., Cunningham, S.A., and Klein, A.M. (2008). Long-term global
291 trends in crop yield and production reveal no current pollination shortage but increasing
292 pollinator dependency. *Curr Biol* 18**,** 1572-1575.
293 Arismendi, N., Bruna, A., Zapata, N., and Vargas, M. (2016). PCR-specific detection of
294 recently described Lotmaria passim (Trypanosomatidae) in Chilean apiaries. *J Invertebr*
295 *Pathol* 134**,** 1-5.
296 Beneke, T., Madden, R., Makin, L., Valli, J., Sunter, J., and Gluenz, E. (2017). A CRISPR Cas9
297 high-throughput genome editing toolkit for kinetoplastids. *Open Science* 4**,** 170095.
298 Brown, M.J., Schmid‐Hempel, R., and Schmid‐Hempel, P. (2003). Strong context‐
299 dependent virulence in a host‐parasite system: reconciling genetic evidence with
300 theory. *Journal of Animal Ecology* 72**,** 994-1002.
301 Canavaci, A.M., Bustamante, J.M., Padilla, A.M., Brandan, C.M.P., Simpson, L.J., Xu, D.,
302 Boehlke, C.L., and Tarleton, R.L. (2010). In vitro and in vivo high-throughput assays
303 for the testing of anti-Trypanosoma cruzi compounds. *PLoS neglected tropical diseases*
304 4**,** e740.
305 Cavigli, I., Daughenbaugh, K.F., Martin, M., Lerch, M., Banner, K., Garcia, E., Brutscher, L.M.,
306 and Flenniken, M.L. (2016). Pathogen prevalence and abundance in honey bee colonies
307 involved in almond pollination. *Apidologie* 47**,** 251-266.
308 Cepero, A., Ravoet, J., Gómez-Moracho, T., Bernal, J.L., Del Nozal, M.J., Bartolomé, C.,
309 Maside, X., Meana, A., González-Porto, A.V., De Graaf, D.C., Martín-Hernández, R.,
310 and Higes, M. (2014). Holistic screening of collapsing honey bee colonies in Spain: a
311 case study. *BMC Research Notes* 7**,** 649.
312 Cersini, A., Antognetti, V., Conti, R., Velletrani, F., and Formato, G. (2015). First PCR
313 isolation of Crithidia mellificae (Euglenozoa: Trypanosomatidae) in Apis mellifera
314 (Hymenoptera: Apidae) in Italy. *Fragmenta entomologica* 47**,** 45-49.
315 Evans, J.D., and Schwarz, R.S. (2011). Bees brought to their knees: microbes affecting honey
316 bee health. *Trends in microbiology* 19**,** 614-620.
317 Gantz, V.M., and Bier, E. (2015). The mutagenic chain reaction: a method for converting
318 heterozygous to homozygous mutations. *Science***,** aaa5945.
319 Gegear, R.J., Otterstatter, M.C., and Thomson, J.D. (2006). Bumble-bee foragers infected by a
320 gut parasite have an impaired ability to utilize floral information. *Proc Biol Sci* 273**,**
321 1073-1078.
322 Goulson, D., Nicholls, E., Botías, C., and Rotheray, E.L. (2015). Bee declines driven by
323 combined stress from parasites, pesticides, and lack of flowers. *Science* 347**,** 1255957.

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324 Klein, A.-M., Vaissiere, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C.,
325 and Tscharntke, T. (2007). Importance of pollinators in changing landscapes for world
326 crops. *Proceedings of the Royal Society of London B: Biological Sciences* 274**,** 303-313.
327 Lander, N., Chiurillo, M.A., Storey, M., Vercesi, A.E., and Docampo, R. (2016).
328 CRISPR/Cas9-mediated endogenous C-terminal tagging of Trypanosoma cruzi genes
329 reveals the acidocalcisome localization of the inositol 1, 4, 5-trisphosphate receptor.
330 *Journal of Biological Chemistry* 291**,** 25505-25515.
331 Lander, N., Chiurillo, M.A., Vercesi, A.E., and Docampo, R. (2017). Endogenous C-terminal
332 tagging by CRISPR/Cas9 in Trypanosoma cruzi. *Bio-protocol* 7.
333 Lander, N., Li, Z.-H., Niyogi, S., and Docampo, R. (2015a). CRISPR/Cas9-induced disruption
334 of paraflagellar rod protein 1 and 2 genes in Trypanosoma cruzi reveals their role in
335 flagellar attachment. *MBio* 6**,** e01012-01015.
336 Lander, N., Li, Z.H., Niyogi, S., and Docampo, R. (2015b). CRISPR/Cas9-Induced Disruption
337 of Paraflagellar Rod Protein 1 and 2 Genes in Trypanosoma cruzi Reveals Their Role in
338 Flagellar Attachment. *MBio* 6**,** e01012.
339 Langridge, D.F., and Mcghee, R.B. (1967). Crithidia mellificae n. sp. an acidophilic
340 trypanosomatid of the honey bee Apis mellifera. *J Protozool* 14**,** 485-487.
341 Martel, D., Beneke, T., Gluenz, E., Späth, G.F., and Rachidi, N. (2017). Characterisation of
342 Casein Kinase 1.1 in Leishmania donovani Using the CRISPR Cas9 Toolkit. *BioMed*
343 *research international* 2017.
344 Morimoto, T., Kojima, Y., Yoshiyama, M., Kimura, K., Yang, B., Peng, G., and Kadowaki, T.
345 (2013). Molecular detection of protozoan parasites infecting Apis mellifera colonies in
346 Japan. *Environmental microbiology reports* 5**,** 74-77.
347 Park, M., Song, K., Reichardt, I., Kim, H., Mayer, U., Stierhof, Y.-D., Hwang, I., and Jürgens,
348 G. (2013). Arabidopsis μ-adaptin subunit AP1M of adaptor protein complex 1 mediates
349 late secretory and vacuolar traffic and is required for growth. *Proceedings of the*
350 *National Academy of Sciences* 110**,** 10318-10323.
351 Passos-Silva, D.G., Rajão, M.A., Nascimento De Aguiar, P.H., Vieira-Da-Rocha, J.P., Machado,
352 C.R., and Furtado, C. (2010). Overview of DNA Repair in Trypanosoma cruzi,
353 Trypanosoma brucei, and Leishmania major. *Journal of nucleic acids* 2010.
354 Peng, D., Kurup, S.P., Yao, P.Y., Minning, T.A., and Tarleton, R.L. (2015).
355 CRISPR-Cas9-mediated single-gene and gene family disruption in Trypanosoma cruzi.
356 *MBio* 6**,** e02097-02014.
357 Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O., and Kunin, W.E. (2010).
358 Global pollinator declines: trends, impacts and drivers. *Trends in ecology & evolution*
359 25**,** 345-353.

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360 Ravoet, J., De Smet, L., Meeus, I., Smagghe, G., Wenseleers, T., and De Graaf, D.C. (2014).
361 Widespread occurrence of honey bee pathogens in solitary bees. *Journal of Invertebrate*
362 *Pathology* 122**,** 55-58.
363 Ravoet, J., Maharramov, J., Meeus, I., De Smet, L., Wenseleers, T., Smagghe, G., and De Graaf,
364 D.C. (2013). Comprehensive bee pathogen screening in Belgium reveals Crithidia
365 mellificae as a new contributory factor to winter mortality. *PLoS One* 8**,** e72443.
366 Ravoet, J., Schwarz, R.S., Descamps, T., Yanez, O., Tozkar, C.O., Martin-Hernandez, R.,
367 Bartolome, C., De Smet, L., Higes, M., Wenseleers, T., Schmid-Hempel, R., Neumann,
368 P., Kadowaki, T., Evans, J.D., and De Graaf, D.C. (2015). Differential diagnosis of the
369 honey bee trypanosomatids Crithidia mellificae and Lotmaria passim. *J Invertebr*
370 *Pathol* 130**,** 21-27.
371 Regan, T., Barnett, M.W., Laetsch, D.R., Bush, S.J., Wragg, D., Budge, G.E., Highet, F., Dainat,
372 B., De Miranda, J.R., and Blaxter, M. (2018). Characterisation of the UK honey bee
373 (Apis mellifera) metagenome. *bioRxiv***,** 293647.
374 Rico, E., Jeacock, L., Kovářová, J., and Horn, D. (2018). Inducible high-efficiency
375 CRISPR-Cas9-targeted gene editing and precision base editing in African trypanosomes.
376 *Scientific Reports* 8**,** 7960.
377 Riddell, C., Sumner, S., Adams, S., and Mallon, E. (2011). Pathways to immunity: temporal
378 dynamics of the bumblebee (Bombus terrestris) immune response against a
379 trypanosomal gut parasite. *Insect molecular biology* 20**,** 529-540.
380 Riddell, C.E., Garces, J.D.L., Adams, S., Barribeau, S.M., Twell, D., and Mallon, E.B. (2014).
381 Differential gene expression and alternative splicing in insect immune specificity. *BMC*
382 *genomics* 15**,** 1031.
383 Runckel, C., Derisi, J., and Flenniken, M.L. (2014). A draft genome of the honey bee
384 trypanosomatid parasite Crithidia mellificae. *PLoS One* 9**,** e95057.
385 Salathe, R., Tognazzo, M., Schmid-Hempel, R., and Schmid-Hempel, P. (2012). Probing
386 mixed-genotype infections I: extraction and cloning of infections from hosts of the
387 trypanosomatid Crithidia bombi. *PLoS One* 7**,** e49046.
388 Schlüns, H., Sadd, B.M., Schmid-Hempel, P., and Crozier, R.H. (2010). Infection with the
389 trypanosome Crithidia bombi and expression of immune-related genes in the bumblebee
390 Bombus terrestris. *Developmental & Comparative Immunology* 34**,** 705-709.
391 Schmid-Hempel, P., Aebi, M., Barribeau, S., Kitajima, T., Du Plessis, L., Schmid-Hempel, R.,
392 and Zoller, S. (2018). The genomes of Crithidia bombi and C. expoeki, common
393 parasites of bumblebees. *PloS one* 13**,** e0189738.

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394 Schmid‐Hempel, R., and Tognazzo, M. (2010). Molecular divergence defines two distinct
395 lineages of Crithidia bombi (Trypanosomatidae), parasites of bumblebees. *Journal of*
396 *Eukaryotic Microbiology* 57**,** 337-345.
397 Schwarz, R.S., Bauchan, G.R., Murphy, C.A., Ravoet, J., De Graaf, D.C., and Evans, J.D.
398 (2015). Characterization of Two Species of Trypanosomatidae from the Honey Bee
399 Apis mellifera: Crithidia mellificae Langridge and McGhee, and Lotmaria passim n.
400 gen., n. sp. *J Eukaryot Microbiol* 62**,** 567-583.
401 Schwarz, R.S., and Evans, J.D. (2013). Single and mixed-species trypanosome and
402 microsporidia infections elicit distinct, ephemeral cellular and humoral immune
403 responses in honey bees. *Developmental & Comparative Immunology* 40**,** 300-310.
404 Sollelis, L., Ghorbal, M., Macpherson, C.R., Martins, R.M., Kuk, N., Crobu, L., Bastien, P.,
405 Scherf, A., Lopez‐Rubio, J.J., and Sterkers, Y. (2015). First efficient CRISPR‐Cas9
406 ‐mediated genome editing in Leishmania parasites. *Cellular microbiology* 17**,**
407 1405-1412.
408 Stevanovic, J., Schwarz, R.S., Vejnovic, B., Evans, J.D., Irwin, R.E., Glavinic, U., and
409 Stanimirovic, Z. (2016). Species-specific diagnostics of Apis mellifera
410 trypanosomatids: a nine-year survey (2007–2015) for trypanosomatids and
411 microsporidians in Serbian honey bees. *Journal of invertebrate pathology* 139**,** 6-11.
412 Vavilova, V., Konopatskaia, I., Luzyanin, S., Woyciechowski, M., and Blinov, A. (2017).
413 Crithidia and Lotmaria in the honeybee and bumblebee populations: a case study in
414 india. *Вавиловский журнал генетики и селекции* 21**,** 943-951.
415 Zhang, W.-W., Lypaczewski, P., and Matlashewski, G. (2017). Optimized CRISPR-Cas9
416 genome editing for Leishmania and its use to target a multigene family, induce
417 chromosomal translocation, and study DNA break repair mechanisms. *mSphere* 2**,**
418 e00340-00316.
419 Zhang, W.W., and Matlashewski, G. (2015). CRISPR-Cas9-Mediated Genome Editing in
420 Leishmania donovani. *MBio* 6**,** e00861.
421
422

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423 **Figure legends**

424

425 **Figure 1 *L. passim* clone expressing tdTomato**

426 All parasites are labelled by red fluorescence in the selected *L. passim* clone expressing
427 tdTomato (0 week, A-C). The parasites keep expressing tdTomato when cultured for 14
428 weeks with G418 (14 weeks +G418, D-F) but not without G418 (14 weeks -G418, G-I).
429 White bar = 30 μm.

430

431 **Figure 2 Cas9 protein expression and growth of *L. passim* clone expressing Cas9**

432 **(A)** The cell lysates of wild type (WT) and FLAG-tagged Cas9 expressing (Cas9) *L.*
433 *passim* were analyzed by western blot using anti-FLAG antibody (upper panel). The
434 same SDS-PAGE gel was stained by coomassie brilliant blue as the loading control
435 (lower panel). The size (kD) of protein molecular weight marker (MW) is at the left. **(B)**
436 Growth of WT (black) and Cas9 (red) *L. passim* in the modified FPFB medium. The
437 experiment was repeated three times and the error bars indicate the standard deviations.

438

439 **Figure 3 Generation of miltefosine transporter knocked out *L. passim* by**
440 **CRISPR/Cas9-induced homology directed repair**

441 **(A)** Schematic representation of the strategy used to generate *L. passim miltefosine*
442 *transporter* knocked out (*LpMT KO*) parasite by CRISPR/Cas9-induced homology
443 directed repair (HDR). The positions of primers to detect wild type (*WT*) and *KO* alleles
444 of *LpMT* are shown with the expected sizes of PCR amplicons. 5' and 3' untranslated
445 regions (UTR) as well as open reading frame (ORF) of *LpMT* are shown in blue, green,
446 and yellow, respectively. Donor DNA contains a hygromycin resistance gene (red)
447 flanked by the parts of 5'UTR and ORF of *LpMT*. The putative cleavage site by Cas9 is
448 at 1,147 bp from the start codon of *LpMT*. 11 antibiotics (blasticidin, G418, and
449 hygromycin) resistant clones (1-11) together with wild type *L. passim* were analyzed by
450 genomic PCR to detect *WT* (851 bp amplicon) and *KO* (1230 bp amplicon) alleles of
451 *LpMT*. The position of 500, 1000, and 3000 bp DNA molecular weight marker (MW) is
452 shown at the left. **(B)** Detection of *LpMT* and *GAPDH* mRNAs in *LpMT* heterozygous
453 (+/-) and homozygous (-/-) knocked-out together with wild type *L. passim* (+/+) by
454 RT-PCR. The expected sizes of RT-PCR amplicons for *LpMT* and *GAPDH* are 564 bp
455 and 279 bp, respectively. The negative control was run using water as the template (-RT)
456 for RT-PCR. The position of 200-700 bp DNA molecular weight marker (MW) is
457 shown at the right.

458

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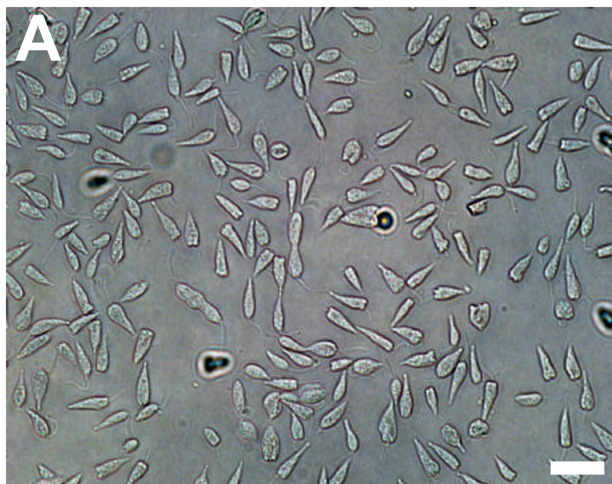
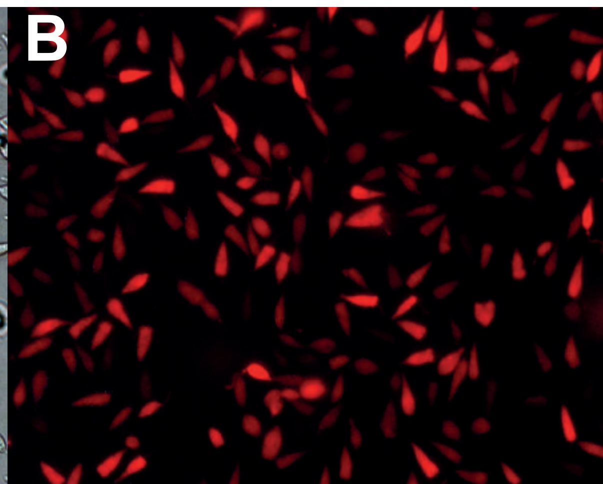
459 **Figure 4 Generation of tyrosine amino transferase knocked out *L. passim* by**
460 **CRISPR/Cas9-induced HDR**
461 **(A)** Schematic representation of the strategy used to generate *L. passim tyrosine amino*
462 *transferase* knocked out (*LpTAT KO*) parasite by CRISPR/Cas9-induced HDR is shown
463 as in Fig. 3A. Donor DNA contains a hygromycin resistance gene (red) flanked by the
464 parts of 5' and 3'UTRs of *LpTAT*. The putative cleavage site by Cas9 is at 111 bp from
465 the start codon of *LpTAT*. 11 antibiotics (blasticidin, G418, and hygromycin) resistant
466 clones (1-11) together with wild type *L. passim* were analyzed by genomic PCR to
467 detect *WT* (1106 bp amplicon) and *KO* (1128 bp amplicon) alleles of *LpTAT*. The
468 position of 500, 1000, and 3000 bp DNA molecular weight marker (MW) is shown at
469 the right. **(B)** Detection of *LpTAT* and *GAPDH* mRNAs in *LpTAT* heterozygous (+/-)
470 and homozygous (-/-) knocked-out together with wild type *L. passim* (+/+) by RT-PCR.
471 The expected sizes of RT-PCR amplicons for *LpTAT* and *GAPDH* are 455 bp and 279
472 bp, respectively. The negative control was run using water as the template (-RT) for
473 RT-PCR. The position of 200-600 bp DNA molecular weight marker (MW) is shown at
474 the right.
475
476 **Figure 5 Growth of *LpTAT* and *LpMT* homozygous knocked out together with wild**
477 **type *L. passim***
478 Growth of *LpTAT* (red) and *LpMT* (green) homozygous knocked out together with wild
479 type (black) *L. passim* in the modified FPFB medium was measured. The experiment
480 was repeated three times and the error bars indicate the standard deviations. Two
481 different clones were tested for each homozygous knocked out parasite.
482
483 **Figure 6 Comparison of knocking out *LpMT* by CRISPR/Cas9-induced HDR and**
484 **homologous recombination**
485 **(A)** Wild type (*WT*) and knocked out (*LpMT KO*) alleles of *LpMT* are shown as in Fig.
486 3A. The positions of primers to detect *LpMT KO* allele are shown with the expected size
487 of PCR amplicon. **(B)** Ten antibiotics (blasticidin, G418, and hygromycin) resistant
488 clones (1-10) isolated by CRISPR/Cas9-induced HDR together with wild type *L. passim*
489 were analyzed by genomic PCR to detect *WT* (851 bp amplicon) and *KO* (803 bp
490 amplicon) alleles of *LpMT*. The position of 500 and 1000 bp DNA molecular weight
491 marker (MW) is shown at the right. **(C)** Ten hygromycin resistant clones (1-10) isolated
492 by homologous recombination were analyzed as in **(B)**


DIC
0 week
A

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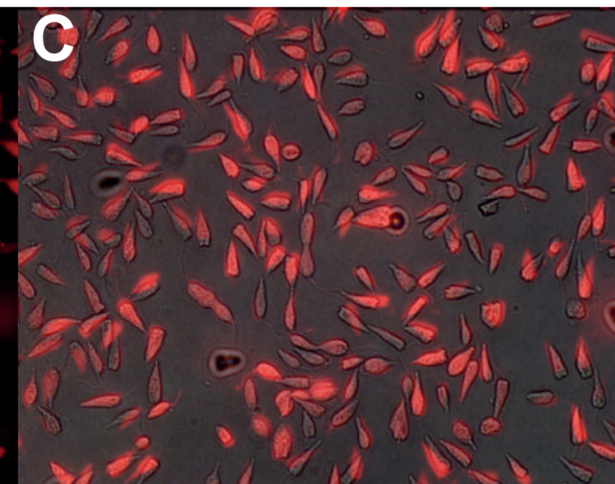
B

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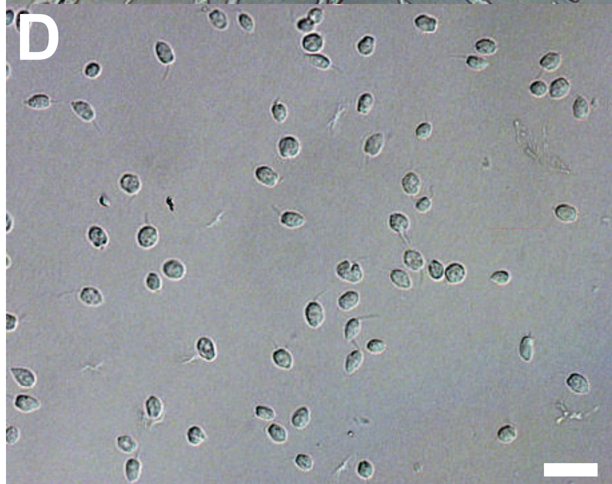
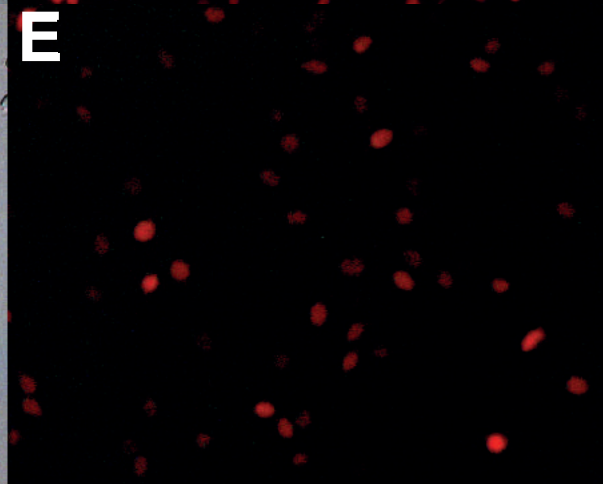
C

14 weeks
+G418
D

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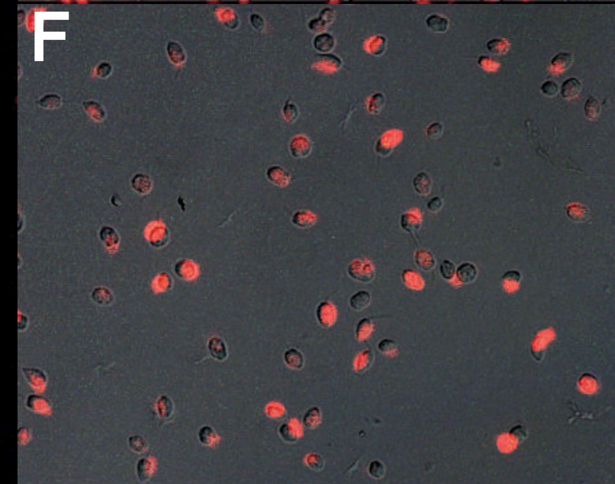
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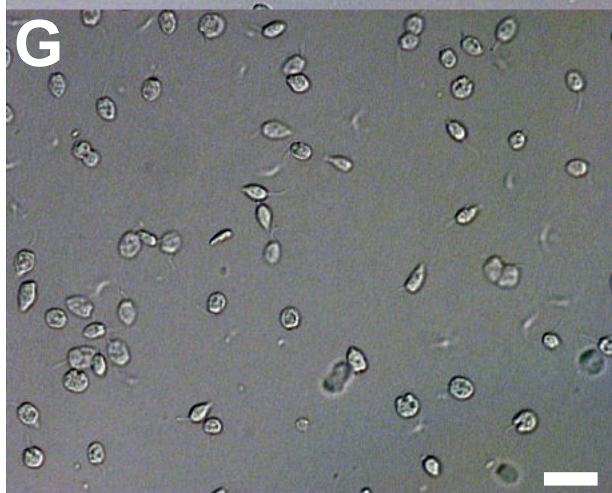
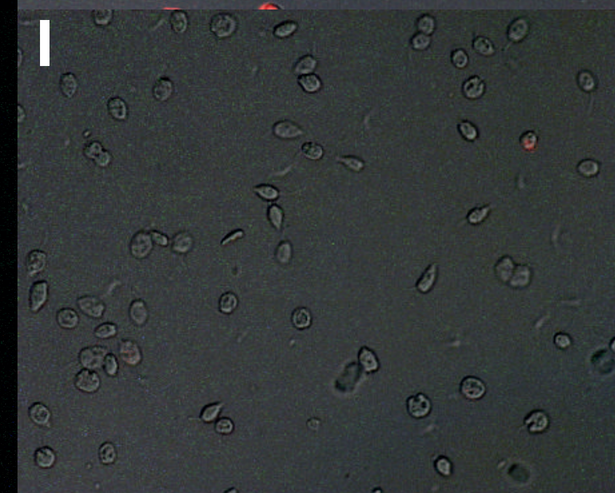
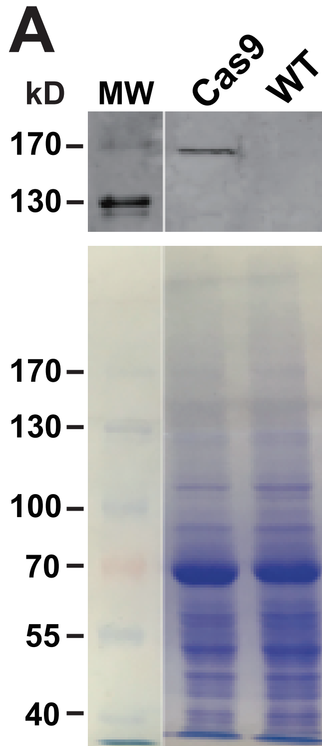

14 weeks
-G418
G

H

I



B

Number of *L. passim* (log)

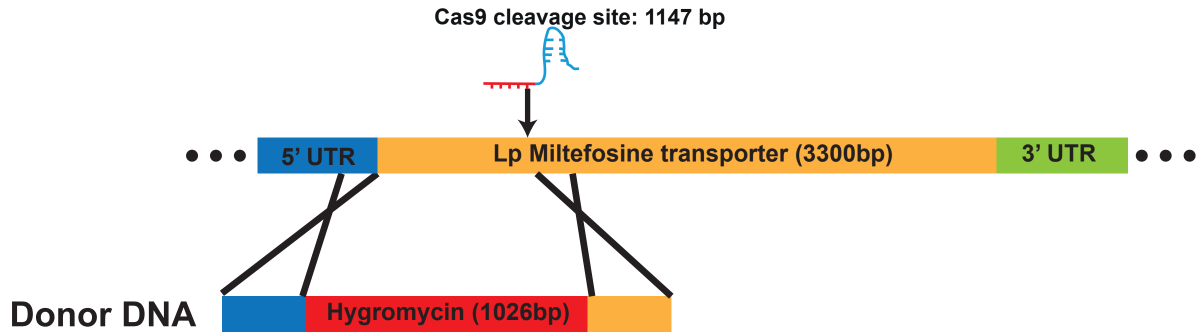
8
7
6
5

0
1
2
3
4
5

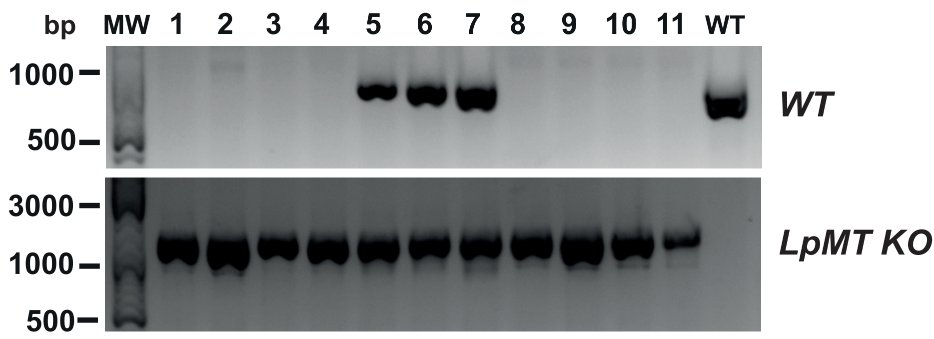
Days

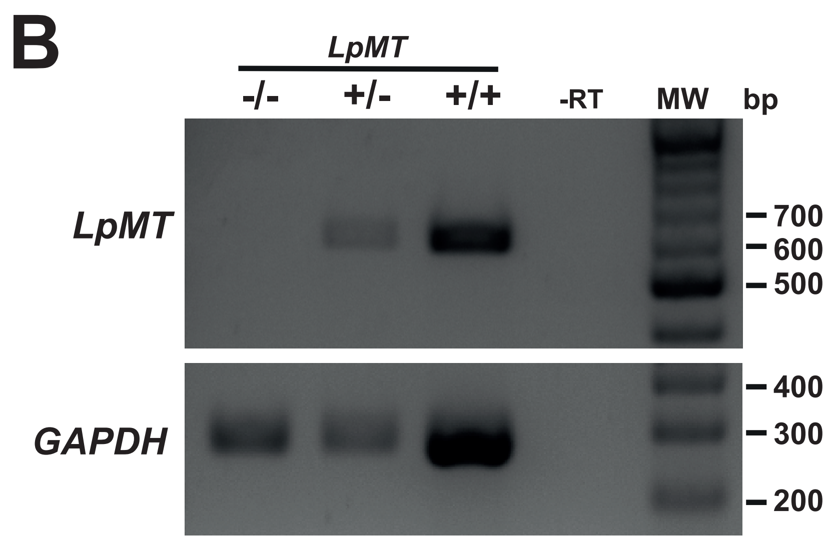
WT
Cas9

A
WT
5' UTR
Lp Miltefosine transporter (3300bp)
3' UTR
851 bp
564 bp


Cas9 cleavage site: 1147 bp
5' UTR
Lp Miltefosine transporter (3300bp)
3' UTR
Donor DNA
Hygromycin (1026bp)

1230 bp
LpMT KO
5' UTR
Hygromycin (1026bp)
Lp Miltefosine transporter (2153bp)
3' UTR


bp
MW
1
2
3
4
5
6
7
8
9
10
11
WT
1000
500
WT
3000
1000
500
LpMT KO


B
LpMT
-/-
+/-
+/+
-RT
MW
bp
LpMT
700
600
500
GAPDH
400
300
200

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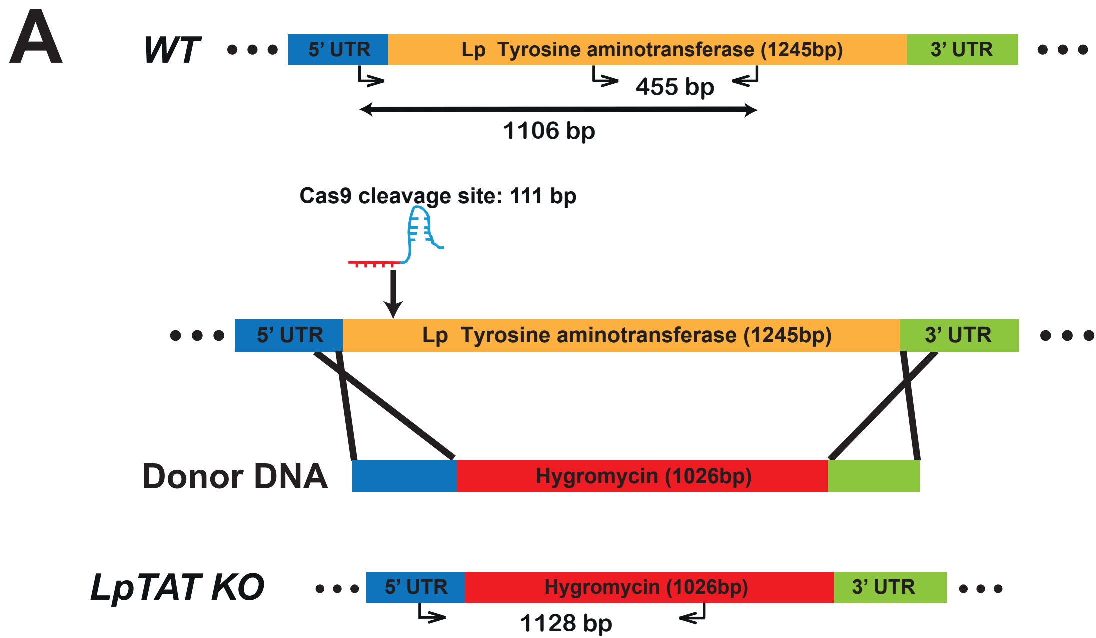

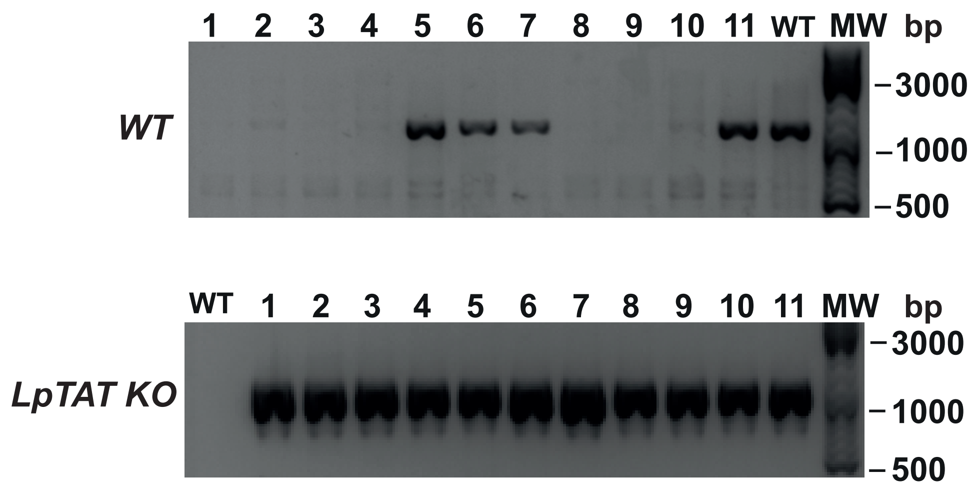

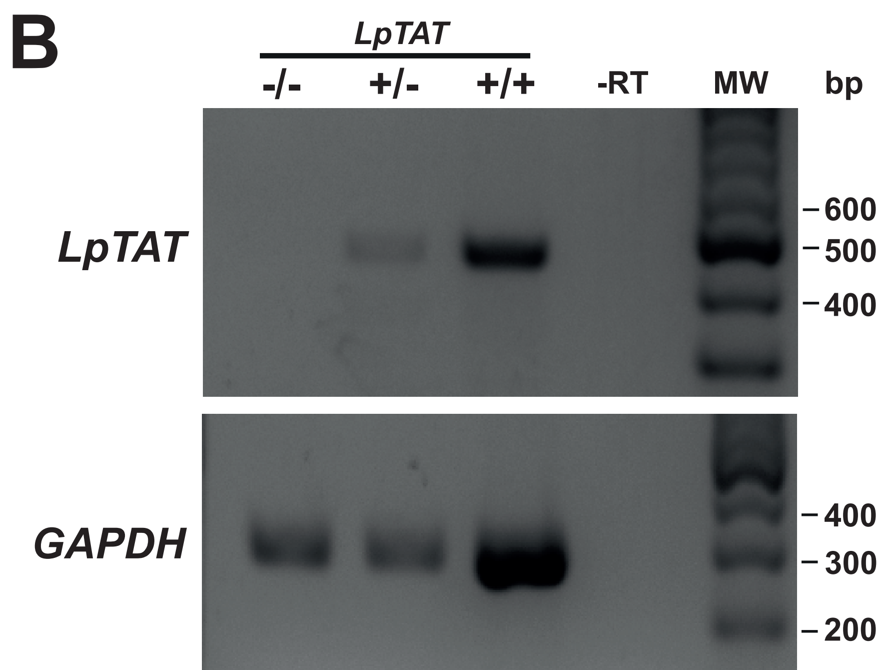

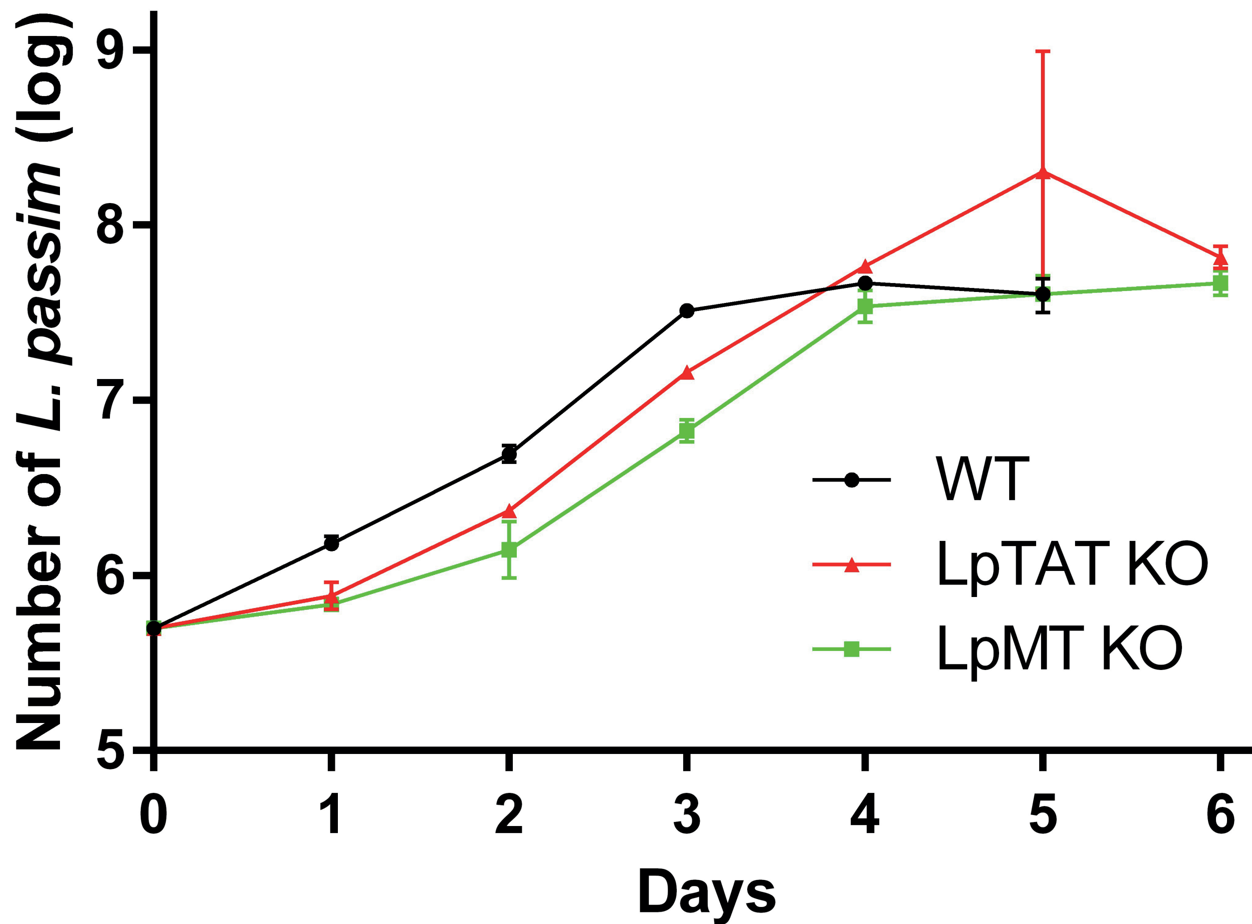

Number of *L. passim* (log)
9
8
7
6
5
0
1
2
3
4
5
6
Days
WT
LpTAT KO
LpMT KO

A

WT
5' UTR
Lp Miltefosine transporter (3300bp)
3' UTR
851 bp

LpMT KO
5' UTR
Hygromycin (1026bp)
Lp Miltefosine transporter (2153bp)
3' UTR
803 bp

B

1 2 3 4 5 6 7 8 9 10 WT MW bp

WT
1000
500

LpMT KO
1000
500

C

1 2 3 4 5 6 7 8 9 10 WT MW bp

WT
1000
500

LpMT KO
1000
500