CRISPR/Cas9-mediated gene deletion of the *ompA gene* in an *Enterobacter* gut symbiont impairs biofilm formation and reduces gut colonization of *Aedes aegypti* mosquitoes

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Running head: CRISPR/Cas9 engineering of a gut bacterium.

Abstract

Symbiotic bacteria are pervasive in mosquitoes and their presence can influence development, reproduction, and immunity of their host. It is evident that environmental and host genetic factors contribute in shaping the microbiome of mosquitoes, but we have a poor understanding regarding how bacterial genetics affects colonization of the mosquito gut. While CRISPR/Cas9 gene editing is a powerful tool to modify bacterial genomes this approach has yet to be applied to insect symbionts. To demonstrate that gene editing can be completed in non-model bacterial species isolated from insects and to investigate the role of bacterial genes in gut colonization, we mutated the outer membrane protein A (*ompA*) gene of an *Enterobacter* symbiont using the CRISPR/Cas9 system. The Δ*ompA* mutant had an impaired ability to form biofilms and poorly infected *Ae. aegypti* when reared in a mono-association under gnotobiotic conditions. In adults, the mutant had a significantly reduced infection prevalence compared to the wild type or complement strains, while no differences in prevalence were seen in larvae, suggesting bacterial genetic factors are important for adult gut colonization. Integration of genes (antibiotic resistance and fluorescent markers) into the symbiont genome demonstrated this technology can be exploited to develop novel symbiotic control strategies to interfere with arboviral pathogens such Chikungunya, Zika and Yellow fever viruses transmitted by *Aedes* mosquitoes. Our results shed insights onto the role of *ompA* gene in host-microbe interactions in *Ae. aegypti* and confirm that CRISPR/Cas9 gene editing can be employed for genetic manipulation of non-model gut microbes.

Importance

CRISPR/Cas9 gene editing approaches have revolutionized several biological fields, however despite their applicability for altering bacterial genomes, few studies use this technology in microbes that associated with eukaryotic hosts. Here we use this editing approach to knockout a gene encoding a membrane protein in an *Enterobacter* isolated from *Aedes* mosquitoes and show this gene is essential for biofilm formation and promotes bacteria colonization of the gut. A reduced bacterial load of the mutant compared to the wild type or complement strains, was seen in both larval and adult mosquitoes, however this was most evident in adults, likely due differences in the mode of acquisition of microbes at each life stage. Our work extends CRISPR/Cas9 genetic manipulation into a new bacterial species, and in conjunction with other studies, suggests that members within *Enterobacteriaceae* are amenable to genome engineering by this approach. This study will facilitate the development of novel microbial-based approaches to mitigate mosquito-borne disease.

Keywords: CRISPR/Cas9, Gut microbe, Gut colonization, Biofilm, Paratransgenesis, Paratransgenic

63 **Introduction.**

64 Mosquitoes harbor a community of microbes within their guts. In general, the gut-
65 associated microbiome of mosquitoes tends to have low species richness but can differ
66 greatly between individuals and habitats[1-8]. Importantly, these microbes can modulate
67 many host phenotypes, several of which can influence vectorial capacity[9-11]. As such, it
68 is imperative that we understand how the microbiome is acquired and maintained within
69 mosquito vectors. While environmental factors unquestionably influences mosquito
70 microbiome composition and abundance[2-4,8], and studies are elucidating the role of
71 microbial interactions[5,7,12,13] and host genetic factors[14-18] in shaping the microbiome, we
72 have a poor understanding regarding bacterial factors that influence colonization of the
73 mosquito gut.

74

75 In other invertebrates, several bacterial genes have been implicated in gut colonization.
76 For example, a genome wide screen exploiting transposon-sequencing found a suite of
77 genes from the bacterium *Snodgrasselia* involved in colonization of the honey bee gut[19].
78 These bacterial genes were classified into the broad categories of extracellular
79 interactions, metabolism and stress response[19]. Knock out of a purine biosynthesis
80 gene in *Burkholderia* impaired biofilm formation and reduced bacterial colonization rates
81 in a bean bug. Biofilm formation was also shown to play a role in virulence of
82 pathogenic *Pseudomonas* in artificial infections of *Drosophila,* with strains that lacked
83 the capacity to form biofilms being more virulence to the host, while a hyperbiofilm strain
84 was less virulent than the WT strain[20]. In other blood feeding invertebrates, bacterial
85 genetics also appears critical for host colonization. Knockout of the type II secretion

system in *Aeromonas veronii* reduced infection in *Hirudo verbena* leeches[21]. In Tsetse flies, the outer-membrane protein A (*ompA*) gene of *Sodalis glossinidius* is essential for symbiotic interactions[22]. *Sodalis* mutants lacking the *ompA* gene poorly colonized the fly gut compared to the wild type (WT) *Sodalis*[22] and the mutant strain also had a reduced capacity to form biofilms[23]. Heterologous expression of the *ompA* gene from pathogenic *Escherichia coli* in *Sodalis* mutants induced mortality in the fly implicating this gene as a virulence factor in pathogenic bacteria[22]. Taken together, these studies suggest that bacterial genetic factors are critical for host colonization of invertebrates and that biofilm formation facilitates symbiotic associations in insects.

In mosquitoes, few studies have investigated how bacterial genetics affect gut colonization. However, evidence from experimental evolution studies suggests bacterial genetics plays a critical role. In two separate studies, *Enterobacter* was selected for increased persistence in the gut of *Anopheles gambiae* mosquitoes, the major malaria vector in sub-Saharan Africa, by repeatedly infecting mosquitoes with strains that persisted in the gut for longer periods of time[24,25]. Transcriptomics comparisons of effective and ineffective colonizers in liquid media identified 41 genes that were differentially expressed between these two strains[25], further implicating the importance of bacterial genetics in mosquito infection, however the role of these genes in colonization of the mosquito gut has not been resolved. In a separate study, *in vitro* screening of a transposon mutant library of *Enterobacter* identified a *waaL* gene mutant that was insensitive to oxidative stress[26]. The *waaL* gene encodes an O antigen ligase which is needed for attachment of the O antigen to lipopolysaccharide and the mutant

109 was found to have lower rates of colonization of the midguts of *Anopheles*
110 mosquitoes[26].
111
112 Gene knockouts approaches in bacteria provide compelling evidence of the role of
113 bacterial genes in host-microbe interactions[21-23,26-28]. In general, most studies use
114 transposon mutagenesis for gene knockout, which requires screening of the mutant
115 library. As *in vivo* screening is burdensome and in some cases not feasible, a targeted
116 gene knockout approach is highly desirable to investigate the functionality of bacterial
117 genes in host-microbe interactions. In the past few years, the CRISPR/Cas9 gene
118 editing system has been employed to modify bacterial genomes[29-31]. While much of the
119 work has been done in model bacterial species[32-36], editing approaches have expanded
120 into non-model bacterial systems[37-42]. Despite this expansion, few studies have used
121 this technology in host-associated microbes[43]. In the vector biology field, gene knockout
122 approaches can be used to interrogate the role of bacterial genes responsible for host-
123 microbe interactions, while the ability to integrate genes into the bacterial symbiont
124 genome has great potential for applied paratransgenic control strategies[11,44-46].
125 Previously, manipulation of non-model symbionts that associate with insect vectors
126 have has been accomplished by plasmid transformation[47-55] or stable transformation of
127 the genome using transposons or integrative plasmids[56-61], but the use of CRISPR/Cas9
128 gene editing in symbionts has yet to be accomplished. For paratransgenic strategies,
129 stable site-specific integration of transgenes into the symbiont genome is critical, and as
130 such, the application of CRISPR/Cas9 gene editing technology to non-model bacteria
131 that associate with insect vectors will stimulate research in this field.

132

133 We therefore undertook studies to develop CRISPR/Cas9 genome editing approaches
134 in an *Enterobacter* species isolated from *Aedes aegypti* mosquitoes, the major vector of
135 arboviruses such as dengue, Zika and Yellow fever viruses. We used the Scarless Cas9
136 Assisted Recombineering (no-SCAR) method to disrupt the *ompA* gene of the non-
137 model *Enterobacter* species[35]. The no-SCAR approach is a single step genome editing
138 system that does not require a selectable marker[35]. After characterization of the mutant
139 *in vitro*, we examined the role of the *ompA* gene in host-microbe interactions by re-
140 infecting bacteria into mosquito in a mono-association. To demonstrate that the
141 CRISPR/Cas9 gene-editing system could be useful for applied symbiotic control
142 approaches we inserted genes conferring antibiotic resistance or a fluorescent protein
143 into the bacterial genome and re-infected the altered strains back into mosquitoes. Our
144 result sheds insights into the role of the *ompA* gene in host-microbe interactions in *Ae.*
145 *aegypti* and confirm that CRISPR/Cas9 gene editing can be a powerful tool for genetic
146 manipulation of native gut-associated microbes of mosquitoes.

147 **Results**

148 ***Enterobacter* biofilm formation in *Ae. aegypti* guts**

149 Over the course of conducting mono-axenic infections in *Ae. aegypti* mosquitoes with
150 an *Enterobacter* symbiont, we repeatedly observed a conglomeration of bacterial cells
151 in the gut that was indicative of a biofilm (Figure 1). This formation of bacteria has a
152 similar appearance to biofilms observed in the guts of other insects[20,23]. No bacteria
153 were observed in *Ae. aegypti* when infections were conducted with *E. coli* that was not
154 adapted to the mosquito gut environment (Figure 1), although as seen previously,
155 infection with *E. coli* enabled mosquito development[62-64]. We therefore sort out to
156 examine the role of bacterial genetics in biofilm formation and host colonization of this
157 gut-associated bacterium of *Aedes* mosquitoes. While several genes have been
158 implicated in biofilm formation[20,23,65], we chose to knockout the *ompA* gene of
159 *Enterobacter* given that this gene has been demonstrated to influence biofilm formation
160 and gut colonization of *Sodalis*[22,23], an *Enterobacteriaceae* symbiont of Tsetse flies,
161 which is phylogenetically related to *Enterobacter*. The CRISRP/Cas9 genome editing
162 system was employed to edit the symbionts genome.

163

164 **Genome editing in non-model *Enterobacter* bacteria isolated from mosquitoes.**

165 To edit the *Enterobacter* isolate that resides within the gut of *Aedes* mosquitoes, we
166 employed the no-SCAR gene editing approach that had been developed in *E. coli*[35]. To
167 optimize the approach in our hands, we performed initial experiments in *E. coli* to delete
168 a ~1 kb region of the *ompA* gene (Figure 2A). As the no-SCAR approach exploits the λ-
169 Red recombineering system to repair double stranded breaks, we supplied cells with a

170 double stranded DNA template that had regions of homology flanking the gRNA site
171 (250 bp for each arm). Using this approach, we successfully deleted a 1001 bp
172 fragment of the *ompA* gene. Of the colonies we screened, we saw an editing at a
173 frequency of 6.25% (N = 48) (Figure 2A). Given our successful editing in *E. coli*, we
174 employed this technique in the non-model *Enterobacter*. However, we altered our
175 editing procedure to delete a 598 bp fragment from the *Enterobacter ompA* gene. This
176 was done to attain a higher frequency of mutants[66] and accommodate a different PAM
177 site in the *ompA* gene of *Enterobacter*. Using a donor template designed for the
178 *Enterobacter ompA* gene that had similar length flanking homology arms as the
179 previous experiment done in *E. coli*, we obtained mutant knockouts at a rate of 32% (N
180 = 50) (Figure 2B). For both bacterial species, Sanger sequencing across the integration
181 site indicated the deletion occurred at the expected loci in the bacterial genome (Figure
182 2C; Supplementary text 1 and 2).

183

184 **Characterization of the *Enterobacter ompA* mutant.**

185 We quantified the growth rates of the Δ*ompA* mutant in comparison to the WT
186 *Enterobacter* and the Δ*ompA/ompA* complement in liquid LB media. We saw no
187 significant difference between the WT, the Δ*ompA* mutant or the Δ*ompA/ompA*
188 complement (Figure 3A). To examine the stability of the deletion, we subcultured the
189 Δ*ompA* mutant on LB media for 10 generations and performed PCR to amplify across
190 the deletion. At alternative generations PCR analysis indicated the deletion was present
191 indicating genomic stability at this site (Figure 3B).

192

193 Previously, *ompA* has been shown to be important in biofilm formation as *Sodalis*
194 deletion mutants were unable to form biofilms[23]. As such we characterized *in vitro*
195 biofilm formation using the crystal violet (CV) biofilm assay. After visual inspection, it
196 was clear the Δ*ompA* mutant had distinctly less biofilm deposition compared to either
197 the WT or the Δ*ompA/ompA* complement (Figure 3C), and after quantification and
198 normalization to account for any difference in growth between the strains, biofilm
199 formation was confirmed to be significantly different between the Δ*ompA* mutant and the
200 WT (Figure 3D; Tukey's multiple comparisons test, $P < 0.0001$) or Δ*ompA/ompA*
201 complement (Tukey's multiple comparisons test, $P < 0.0001$), while the was no
202 significant differences between the WT and the Δ*ompA/ompA* complement (Tukey's
203 multiple comparisons test $P = 0.2$).
204

205 **The role of *ompA* gene in mosquito infection.**

206 To examine the importance of the *ompA* gene on bacterial colonization of mosquitoes,
207 we infected *Ae. aegypti* mosquitoes in a mono-association under gnotobiotic
208 conditions[6]. This infection method was used to avoid other gut-associated microbes
209 influencing host colonization rates[7] and it also assisted in quantification of introduced
210 bacteria by measuring colony forming units (CFUs). In larvae we saw a significant
211 reduction in bacterial titer in the mutant compared to both the WT (Kruskal-Wallis test; P
212 < 0.01) and the Δ*ompA/ompA* complement (Kruskal-Wallis test; $P < 0.05$) (Figure 4A).
213 Similarly, in adults, there was a significant reduction in bacterial infection in the Δ*ompA*
214 mutant compare to either the WT or Δ*ompA/ompA* complement (Kruskal-Wallis test; $P <$
215 0.001) (Figure 4B). While no significant changes were seen in the prevalence of

216 infection (number of mosquitoes infected) in the larval stage (Figure 4C, Fisher's exact
217 test; WT compared to Δ*ompA* P = 0.24 and Δ*ompA* compared to Δ*ompA/ompA* P =
218 0.24), in adults, the prevalence of infection was significantly different (Figure 4D,
219 Fisher's exact test; WT compared to Δ*ompA* P < 0.0001 and Δ*ompA* compared to
220 Δ*ompA/ompA* P < 0.0001), with only 45% of adults infected by the Δ*ompA* mutant
221 compared to 95% and 88% by the WT and Δ*ompA/ompA* complement, respectively. We
222 also examined the growth rates of mosquitoes administered with the WT, Δ*ompA*
223 mutant and Δ*ompA/ompA* complement. No significant differences were seen in the time
224 to pupation (Figure 5A) or percentage of first instar larvae that reached adulthood
225 (Figure 5B) between any of the strains.
226
227 **Integration of genes into the *Enterobacter* chromosome.**
228 We undertook experiments to demonstrate the CRISPR/Cas9 gene-editing approaches
229 can be used to integrate genes into the chromosome of non-model bacteria that
230 associate with mosquitoes. We created two independent transgenic strains that had
231 either, a gene encoding mCherry fluorescence or a gene encoding resistance to the
232 antibiotic gentamicin, inserted into the bacterial chromosome. These genes were
233 integrated into the genome using the same gRNA that was used for deletional
234 mutagenesis, and as such, these insertions also disrupted the *ompA* gene. Sequencing
235 across the integration site indicated the insertion of these genes occurred within the
236 *ompA* gene and thereby disrupted its function (Figure 6A and 6D). Continual
237 subculturing was undertaken for both strains and molecular analysis indicated the
238 stability of these lines for ten generations (Figure 6B and 6E). To demonstrate the

239 integrated genes were functional, we observed expression of mCherry fluorescence and
240 successfully cultured the strain containing gentamicin resistance on plates containing
241 the antibiotic (Figure 6C and 6F). Finally, we infected these transgenic strains into
242 mosquitoes to demonstrate that these strains were able to colonize the mosquito gut
243 and functionality of the integrated gene was confirmed by observing fluorescence or by
244 rearing the *Enterobacter ompA*::gentamicin strain in mosquitoes administered sugar
245 supplemented with gentamicin. Fluorescent bacteria were observed in the gut of
246 mosquitoes while no signal was seen in controls (WT *Enterobacter* infected mosquitoes)
247 (Figure 5G). The *Enterobacter ompA*::gentamicin was successfully rescued from
248 mosquitoes reared on gentamicin and was seen to stably infect mosquitoes over time at
249 a density of $1x10^4$ CFUs/mosquito. Consistent with our previous finding (Figure 4B), the
250 WT bacteria initially infected mosquitoes at higher titers (T test; day 0 $P < 0.001$).
251 However, at 4 days post infection (dpi), the total bacterial load of culturable microbes in
252 mosquitoes supplemented with WT *Enterobacter* was significantly reduced when reared
253 on sugar supplemented with antibiotic (T test; day 4 $P < 0.05$), and no CFUs were
254 recovered after at 6 dpi (T test; day 6 $P < 0.001$) (Figure 6H).

255 **Discussion.**

256 We harnessed the CRISPR/Cas9 gene editing system to create knockout mutants in an
257 *Enterobacter* gut symbiont of *Ae. aegypti* mosquitoes enabling us to examine the role of
258 bacterial genetics, specifically the *ompA* gene, in biofilm formation and gut colonization.
259 A deletion of the *ompA* gene of *Enterobacter* decreased bacterial colonization of the
260 mosquito host at both the larval and adult stages after infection in a mono-association.
261 Strikingly, we found this effect was most pronounced in adult mosquitoes with more
262 than half of the mosquitoes not possessing any culturable mutants, while there was no
263 difference in prevalence of infection between the mutant and WT bacteria in larvae. The
264 reduced prevalence of mutant bacteria in adults likely reflects differences in microbial
265 colonization of each mosquito life stage. Larvae are continually subjected to bacteria in
266 the larval water habitat while adults only have a short time frame to acquire bacteria
267 immediately after eclosion, when they are thought to imbibe a small amount of larval
268 water which seeds the gut with microbiota[67]. Our data shows greater variation in
269 colonization of the adult stage between the mutant and WT strains, indicating that the
270 *ompA* gene, and potentially bacterial factors in general, may be critical for colonization
271 of the adult gut. These findings are also consistent with other sequence-based studies,
272 that indicate adult stages have greater variability in species composition of their
273 microbiota, while the microbiome of immature stages reflects the larval water habitat[1-8].
274

275 Overall, our findings are similar to studies done in Tsetse flies whereby an *ompA* mutant
276 of *Sodalis*, an *Enterobacteriaceae* symbiont, has impaired biofilm formation and
277 reduced colonization rates[22,23]. These studies, in conjunction with our work, suggests

278 that the *ompA* gene is imperative for symbiotic associations within dipterans. It also
279 suggests that biofilm formation may be a strategy employed by bacteria to colonize the
280 gut of insects. In pathogenic infections in mammals, biofilms enable bacteria to colonize
281 new niches, promote infection and are associated with virulence[68,69]. Although less is
282 known regarding the importance of biofilm formation in insects, in an artificial
283 *Pseudomonas-Drosophila* infection model, biofilm formation was associated with
284 virulence and host survival[20]. In a natural symbiotic association between bean bugs and
285 *Burkholderia*, disruption of a purine biosynthesis gene in the bacterium also reduce
286 biofilm formation and colonization of the insect[65]. In mosquitoes, gut biofilm formation
287 could also have implications for vector competence as *Chromobacterium*, which was
288 isolated from *Aedes* mosquitoes, produced molecules that inhibited dengue virus only
289 when grown *in vitro* as a biofilm and not when grown in a planktonic state[70]. However, it
290 is unknown if biofilm formation occurred *in vivo*[70]. Our data provide evidence that
291 biofilms occur within the gut of mosquitoes and facilitate host colonization.
292
293 While we have shown that the *ompA* gene of *Enterobacter* is important for host
294 colonization, we see no evidence that deletion of this gene alters mosquito development
295 or growth rates. This is in contrast to the *Riptortus-Burkholderia* symbiosis whereby
296 mutation of the *purT* gene in *Burkholderia* resulted in reduced growth rates and
297 reduction in body weight of the host compared to insects that were infected with the WT
298 bacterium[65]. The difference in our study to the findings in the *Riptortus-Burkholderia*
299 symbiosis could be related to different requirements of the bean bug compared to the
300 mosquito host as well as the different genes mutated in the symbionts. Our findings are

301 consistent with another study in mosquitoes whereby an *ompA* mutant of *E. coli* was
302 reported to not influence growth when a genomic mutant library was screened in *Ae.*
303 *aegypti* in a mono-association[71]. Using a similar gnotobiotic system that exploits the
304 ability to sterilize mosquito eggs and rescue development of the insect by
305 supplementation, several recent reports describe approaches to create bacteria-free
306 mosquitoes[62,63]. Here, we reared mosquitoes in a mono-association where they were
307 only subjected to *Enterobacter.* However, more than half the adult mosquitoes
308 inoculated with the Δ*ompA* mutant were not infected by bacteria, but nevertheless, had
309 similar development and growth rates compared to mosquito possessing WT bacteria. It
310 would be intriguing to determine if these uninfected mosquitoes were bacteria-free. If
311 so, the use of mutant bacteria that rescue development but cannot colonize mosquitoes
312 may provide a simple means to create sterile bacteria-free mosquitoes.
313
314 CRISPR/Cas9 gene editing has revolutionized genetic approaches in model bacteria[32-
315 35], and while the use of this approach is expanding to other non-model bacteria[37-42], to
316 our knowledge, there are no examples of editing undertaken in symbiotic microbes.
317 Here we demonstrate that editing approaches functional in *E. coli* can be applied to
318 phylogenetically related symbiotic bacteria that associate within the guts of mosquitoes.
319 Our overall goal was to delete the *ompA* gene in *Enterobacter* and therefore we altered
320 our editing strategy when carrying out experiments in the non-model *Enterobacter*
321 compared to our initial attempts in *E. coli*, and as such it is difficult to compare rates of
322 editing between bacterial species. Nevertheless, the number of mutant colonies was
323 considerably greater in *Enterobacter* compared to *E. coli* indicating the editing approach

324 is applicable to this symbiotic *Enterobacteriaceae* bacterium. Similar to our findings, a
325 two-plasmid CRISPR/Cas9 system, exploiting the use of λ-Red recombineering,
326 developed in *E. coli* was also functional in the *Enterobacteriaceae, Taumella citrea*,
327 without the need for modification[36]. Taken together, these findings suggest that
328 CRISPR/Cas9-based approaches may be applicable to a wide range of
329 *Enterobacteriaceae.* Similar to the original study in *E. coli*[35], we observed
330 counterselection escapees in our editing experiments. The no-SCAR approach uses a
331 counterselectoin method to edit bacteria and cells that possess both the Cas9 and the
332 gRNA should not be viable as a result of double stranded breaks in their genome. As
333 the genetic basis of counterselection escape remains unclear and our primary objective
334 was to create a mutant strain, we did not pursue the mechanism behind these escapees
335 further.
336
337 Previous integration attempts in *E. coli* using the no-SCAR approach inserted a small
338 fragment (80 bp) into the bacterial genome[35]. It was hypothesized however, that this
339 approach could be used to integrate larger sized fragments over 1 kb into the genome
340 based on the efficiency of integration and counterselection escape rates, although
341 screening would be required[35]. Here we demonstrate this is indeed feasible as we
342 inserted 1.3 kb and 1.5 kb fragments into the *Enterobacter* genome, and importantly,
343 these genes, driven by the AmTr promoter[72], were functional both *in vitro* and *in vivo*.
344 Our work expands the list of bacteria amenable to CRISPR/Cas9 based genome editing
345 approaches and provides an elegant tool to investigate specific bacterial genes that
346 influence host-microbe interactions in mosquito vectors.

347

348 The application of CRISPR/Cas9 genome editing to gut-associated bacteria of
349 mosquitoes has significant applied potential. Paratransgenesis strategies are being
350 evaluated in a range of medical and agricultural systems to mitigate pathogen
351 transmission from insect vectors, however, most approaches engineer symbionts by
352 plasmid transformation[47-55] and where genome integration has been accomplished in
353 symbionts[56-61], it has been done with technologies that did not allow for site specific
354 integration. Here, we demonstrate site-specific integration of transgenes into the
355 bacterial symbiont genome. Paratransgenic approaches suitable for use in the field will
356 need to stably integrate genes into the bacterial genome in a manner that does not
357 compromise bacterial fitness. As such, the use of CRISPR/Cas9 to engineer specific
358 sites in the bacterial genome, such as intergenic regions within the genome, will
359 undoubtedly be beneficial for these applied approaches.

360

361 In summary, we have demonstrated that the CRISPR/Cas9 gene editing system can be
362 applied to non-model symbiotic bacteria that associate with eukaryotic hosts to
363 interrogate the role of bacterial genes in host-microbe associations. We created
364 knockout and knockin mutants by deleting and disrupting the *ompA* gene of
365 *Enterobacter*. The knockout mutant displayed a reduced ability to form biofilms and
366 colonize the gut of *Ae. aegypti* mosquitoes in a mono-association, demonstrating
367 bacterial genetic factors are important determinants that influence colonization of
368 mosquito guts. *Aedes* mosquitoes are becoming powerful systems to investigate the
369 genetics of host-microbe interactions given the scientific community has simple and

370 efficient approaches to alter both the microbes (this study) and mosquito host
371 genome[73,74] at their disposal, as well as methods to create mono-associated bacterial
372 lines[64]. Finally, rapid, efficient, and site specific gene editing approaches for gut bacteria
373 that associate with mosquitoes will facilitate the development of novel paratransgenic
374 approaches to control arthropod-borne disease.

375 ## Materials and Methods

376 **Bacterial and mosquito strains.** *E. coli* BL21(DE3) (NEB) and an *Enterobacter* strain
377 previous isolated from a lab-reared colony of *Ae. albopictus* (Galveston) mosquitoes[7]
378 were used in this study. Cultures were grown in liquid LB media at 37°C with the
379 appropriate antibiotic unless stated otherwise. Mosquitoes were reared in the UTMB
380 insectary under conventional conditions or in mono-associations (described below).
381

382 **CRISPR gene editing.** Editing the *ompA* gene of *E. coli* and *Enterobacter*
383 (Supplementary Table 1) was complete as described in Reisch and Prather[35]. The
384 photospacer sequence for the *ompA* gene was designed using the CHOPCHOP[75], and
385 cloned into pKDsgRNA-ack plasmid[35] directly upstream of gRNA scaffold using
386 REPLACR mutagenesis protocol[76] (Supplementary Table 2). The plasmids were
387 acquired from Addgene (Supplementary Table 1; Addgene plasmid 62655 and 62654).
388 The resulting plasmids pKDsgRNA-Ec-*ompA* and pKDsgRNA-Ent-*ompA* were Sanger
389 sequenced to confirm insertion of photospacer sequence. These plasmids were then
390 transformed into either *E. coli* or *Enterobacter* containing the pCas9-CR4 plasmid.
391 Transformants were selected at 30°C on LB agar plate containing spectinomycin (50
392 µg/mL), chloramphenicol (34 µg/mL), and with or without anhydrotetracycline (aTC) at
393 100ng/mL. Colonies from the –aTC plate were grown overnight in LB broth with the
394 appropriate antibiotic at 30°C. A 1:100 diluted overnight culture was (grown until 0.4
395 OD_{600}) supplemented with 1.2% arabinose to induce the expression of λ-Red
396 recombinase. Cells were then transformed with 1-1.5 µg of double stranded donor DNA
397 that flanked the PAM site for homologous recombination. Donor DNA was created by

398 either PCR amplification or by gene synthesis (Genewiz). Regardless of the method of
399 construction, each donor had flanking regions of 250 bp homologous to the target DNA.
400 The resulting colonies were screened for mutations by colony PCR with primers flanking
401 the integration site and positive clones were Sanger sequenced. Positive colonies were
402 grown in LB broth and genomic DNA was isolated. For further validation, the flanking
403 regions of deletion or insertions were amplified and the PCR product Sanger
404 sequenced.
405
406 **Stability of insertion.** The stability of the knockout Δ*ompA* mutant and the knockin
407 *ompA*::gentamicin and *ompA*::mCherry strains was assessed in LB medium. The
408 *omp*A::mCherry and knockout Δ*ompA* mutant cultures were grown for 10 passages in
409 LB broth. At each passage 40 µl of culture was transferred into 4ml fresh LB medium.
410 The *ompA*::gentamicin strain was grown with or without gentamicin (50 µg/mL).
411 Genomic DNA was isolated from the 0, 2, 4, 6, 8 and 10th subculture and PCR that
412 amplified across the integration site was performed.
413
414 **Complementation of *ompA* mutant.** Functional rescue of the *omp*A mutation was
415 achieved by complementing the mutant with the WT gene. The WT *omp*A gene was
416 amplified from *Enterobacter* genomic DNA and cloned into the pRAM-mCherry vector[7]
417 and thereby creating pRAM-mCherry-*Ent-OmpA.* The Sanger sequence-verified
418 plasmid was transformed into the Δ*ompA* mutant, thereby generating the Δ*ompA/ompA*
419 complement strain. Colonies that acquired the plasmid were selected on LB plates
420 containing kanamycin (50 µg/mL).

421

422 ***In vitro* characterization of *Enterobacter* strains.** To assess the impact of the gene
423 deletion on bacterial growth the WT, Δ*ompA* mutant and Δ*ompA/ompA* complement
424 were grown in LB broth and the density of bacteria (OD_{600}) was quantified by
425 spectrophotometer. A 1:100 dilution of an overnight culture was inoculated into a 5 ml
426 LB broth in 50 ml tube and incubated at 37°C for 24 hrs. At 2, 4, 6, 8, 10, 12 and 24
427 hours growth was recorded at $OD_{600.}$ The biofilm assay was performed as described
428 previously[77,78]. Briefly, biofilm formation by *Enterobacter* strains was quantified on
429 polystyrene microtiter plates after 72 h of incubation at 37°C by CV staining. Three
430 independent experiments were performed, and the data were represented as CV OD_{570}
431 after normalizing by CFUs.

432

433 **Mosquito infections.** Mono-association in *Ae. aegypti* mosquitoes were done using
434 gnotobiotic infection procedure[64], with slight modifications[7]. Briefly, mosquito eggs were
435 sterilized for 5 min in 70% ethanol, 3 min 3% bleach+0.01% Coverage Plus NPD (Steris
436 Corp.), 5 min in 70% ethanol then rinsed three times in sterile water. Eggs were
437 vacuumed hatched for 30-45 min and left overnight at room temperature to hatch any
438 remaining eggs. Exactly twenty L1 larvae were transferred to T175 flask containing 60
439 ml of sterile water and fed on alternative days with 60 µl of fish food (1 µg/µl). Larvae
440 were inoculated with $1x10^7$/ml of either the WT *Enterobacter*, the Δ*ompA* mutant or the
441 Δ*ompA/ompA* complement. The WT and Δ*ompA* strains were transformed with the
442 pRAM-mCherry plasmid that conferred resistance to kanamycin (but did not possess a
443 functional *ompA* gene). L4 larvae were collected, washed three times with 1X PBS, and

444 then homogenized in 500 µl of 1X PBS and 50 µl of homogenate was plated on LB agar
445 containing 50 µg/mL kanamycin. Similarly, adult mosquitoes were collected 3-4 days
446 post emergence and bacterial infection was quantified in the same manner as larvae. In
447 order to assess the growth of the mosquitoes, time to pupation and growth rate were
448 observed. Time to pupation was determined by quantifying the number of pupae each
449 day post hatching, while survival to adulthood was calculated by quantifying the number
450 of L1 larvae that reached adulthood. The experiment was repeated three times.
451
452 Knock-in mutants were administered to adult *Ae. aegypti* in a sugar meal. Three to four
453 day old mosquitoes were fed with $1x10^7$ of WT or the Δ*ompA*::gentamicin strain for
454 three days in 10% sucrose solution. After three days, mosquitoes were either
455 administered sugar supplemented with gentamicin (50 µg/mL) or sugar without
456 antibiotic. CFUs were determined at days 0, 2, 4, and 6 dpi by plating homogenized
457 mosquitoes (N=10) on LB agar. Similarly, the Δ*ompA*::mCherry and WT *Enterobacter*
458 were fed to mosquitoes and midguts were dissected to assess the colonization of
459 bacteria in the tissue. For visualization of bacteria, midguts were fixed in 1%
460 paraformaldehyde (PFA) in 1X PBS for 30 minutes and permeabilized with 0.01% Triton
461 X-100 in 1X PBS for 20 min. The tissues were stained with 1:250 diluted Phalloidin
462 (Sigma) for 20 minutes and samples were washed twice with 1X PBS for 10 minutes.
463 Finally, midguts were then stained with 1:500 diluted DAPI (Invitrogen) for 10 min.
464 Samples were transferred to slides and mounted with ProLong™ Gold Antifade
465 (Invitrogen). The slides were observed under Revolve FL (ECHOLAB).
466

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477

478 **Competing interests**.

479 The authors declare no competing interests.

480

481 **Figure legends.**

482

483 **Figure 1. Midgut infection of *Enterobacter* and *E. coli* in mono-associations of**
484 ***Aedes* mosquitoes.** *Enterobacter* forms a biofilm in the gut of *Aedes aegypti*
485 mosquitoes (left) while no bacteria were observed in the gut of mosquitoes reared with
486 *E. coli* under gnotobiotic conditions (right). Bacteria possessed a plasmid expressing
487 mCherry. Blue – host nuclei. Green – host actin cytoskeleton stained with phalloidin.
488 The scale bar is 70 µm.

489

490 **Figure 2. CRISPR/Cas9 genome editing in bacteria.** A schematic of the editing
491 approach and screening of putative mutants in *E. coli* (A) and *Enterobacter* (B). A ~1kb
492 fragment of *E. coli* BL21(DE3) was deleted using no-SCAR protocol. The 250 bp of left
493 arm (LA) and right arm (RA) was assembled to generate 500 bp donor DNA. The
494 transformants were screened via colony PCR with primers binding in regions flanking
495 the deletion. Similar to strategy employed in *E. coli*, knockout of the *ompA* gene from
496 *Enterobacter* isolated from the mosquito gut was created by deleting the 598 bp
497 fragment. The green star indicates the PAM site in the *ompA* gene. (C) The sequence of
498 the *ompA* mutation in *E. coli* and *Enterobacter* was confirmed by Sanger sequencing.
499 The sequence above the gene within the dotted line has been deleted. The
500 chromatogram shows the 10 bp flanking the deletion.

501

502 **Figure 3. In vitro characterization of the *ompA* mutation.** The *Enterobacter* Δ*ompA*
503 mutant had a similar growth rate compared to both the WT and the Δ*ompA/ompA*

504 complement in liquid LB media (A). The stability of mutant was evaluated *in vitro* by
505 continuous subculturing in LB medium (B). Genomic DNA of alternative subcultures was
506 used as template for PCR using gene specific primers that amplified across the
507 deletion. Two separate gel images were merged to create the figure 2B. Passage 8 was
508 run on a separate gel to passages 0 – 6. Biofilm formation was assessed using the CV
509 biofilm assay for the WT, Δ*ompA* mutant and the Δ*ompA/ompA* complement (C).
510 Quantification of the relative biofilm formation normalized by the number of bacteria per
511 well (D).
512
513 **Figure 4. The Δ*ompA* mutant poorly infected mosquitoes.** Infection of *Enterobacter*
514 strains (WT, Δ*ompA* mutant and Δ*ompA/ompA* complement) reared in a mono-
515 association using a gnotobiotic rearing approach for larvae (A) and adults (B). L4 and 3-
516 4 days post emergence adults were screened for bacterial load by plating on LB media
517 to quantify the bacteria. The prevalence of infection (number of mosquitoes infected)
518 between the treatments was calculated comparing number of infected to uninfected
519 larvae (C) or adults (D).
520
521 **Figure 5. The Δ*ompA* mutant does not affect growth rates or development of**
522 **mosquitoes.** The growth rate (time to pupation) (A) and development (percentage of L1
523 larvae to reach adulthood) (B) was observed in mosquitoes infected with *Enterobacter*
524 strains (WT, Δ*ompA* mutant and Δ*ompA/ompA* complement) reared in a mono-
525 association.
526

527 **Figure 6. Integration of mCherry and gentamicin into the *Enterobacter* genome.**
528 Sanger sequence across the integration site, stability of the inserted gene and *in vitro*
529 expression of the inserted gene for the Δ*ompA*::mCherry (A-C) and the Δ*ompA*::
530 gentamicin (D-F) strains. The chromatogram shows the sequence spanning the inserted
531 sites. Strains were continually subcultured for 10 passages and PCR was done to
532 examine the stability of the insert (B; Δ*ompA*::mCherry plus WT; E Δ*ompA*::gentamicin
533 passaged with (ab+) or without (ab-) gentamicin plus WT). mCherry fluorescence or
534 ability to grow on selective media containing gentamicin confirmed the expression of the
535 transgene *in vitro*. Mosquitoes were inoculated with the *Enterobacter* strains to confirm
536 expression of the transgene *in vivo*. Dissected midgut infected with Δ*ompA*::mCherry
537 (left) or negative control (right; WT bacteria without expression plasmid) (G). Midguts
538 were stained with phalloidin (green) and DAPI (blue). The scale bar is 30 μM. The WT
539 and Δ*ompA*::gentamicin *Enterobacter* strains were fed to adult mosquitoes for 3 days in
540 a sugar meal before gentamicin was administered to mosquitoes in a sugar meal (H).
541 Mosquitoes were collected every second day and CFUs assessed. Pairwise
542 comparisons were conducted at each time point using a T test (* - $P < 0.05$, *** $P <$
543 0.001, **** $P < 0.0001$).
544

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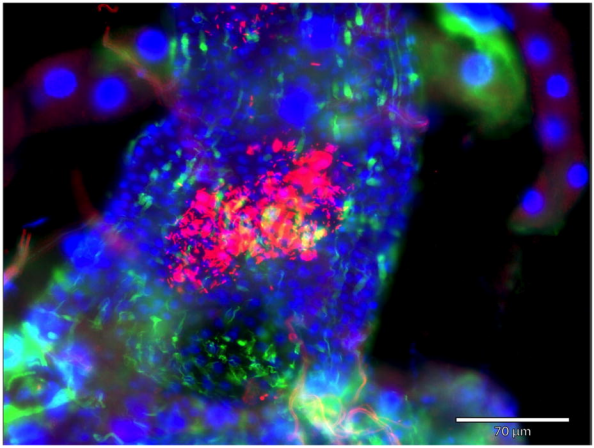
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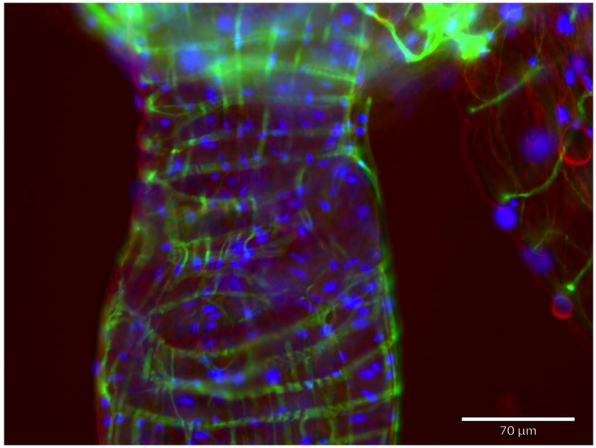
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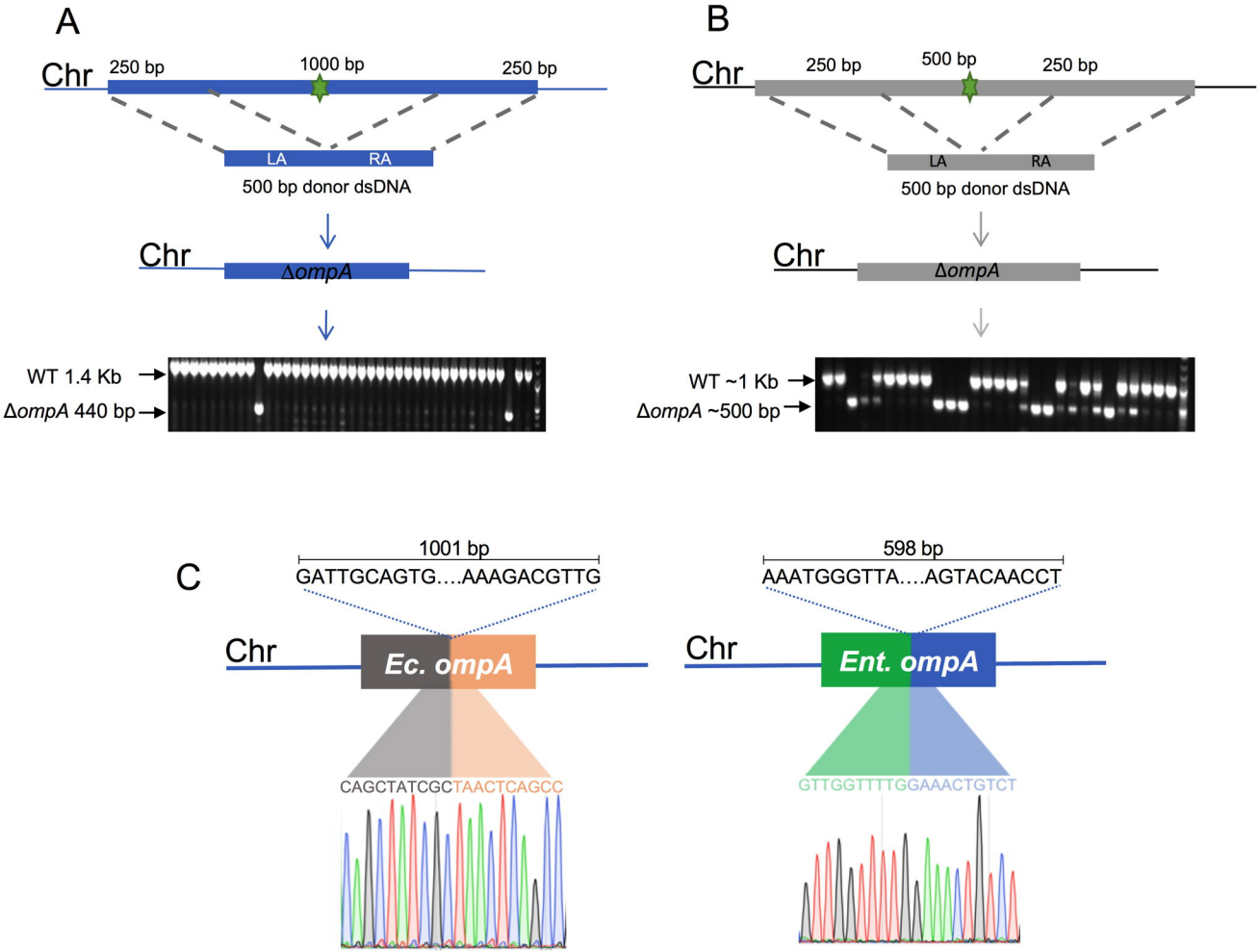
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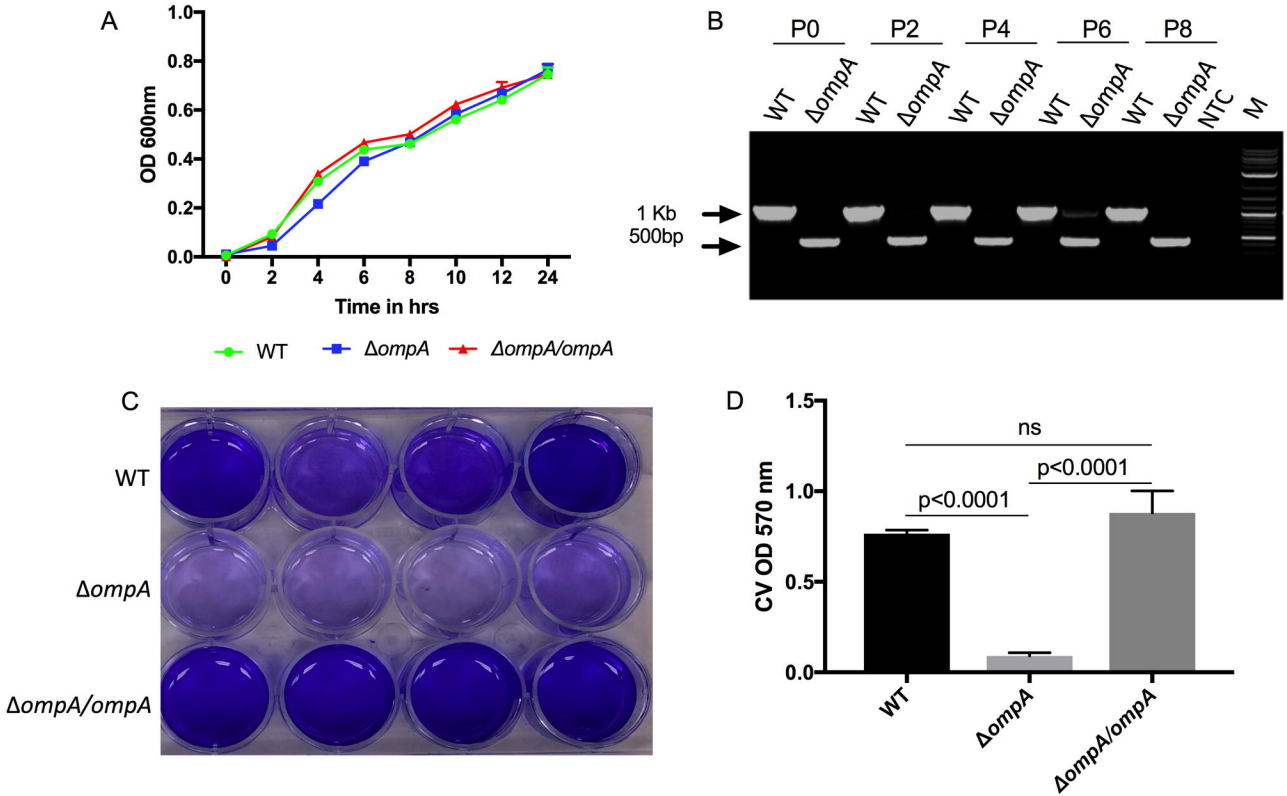
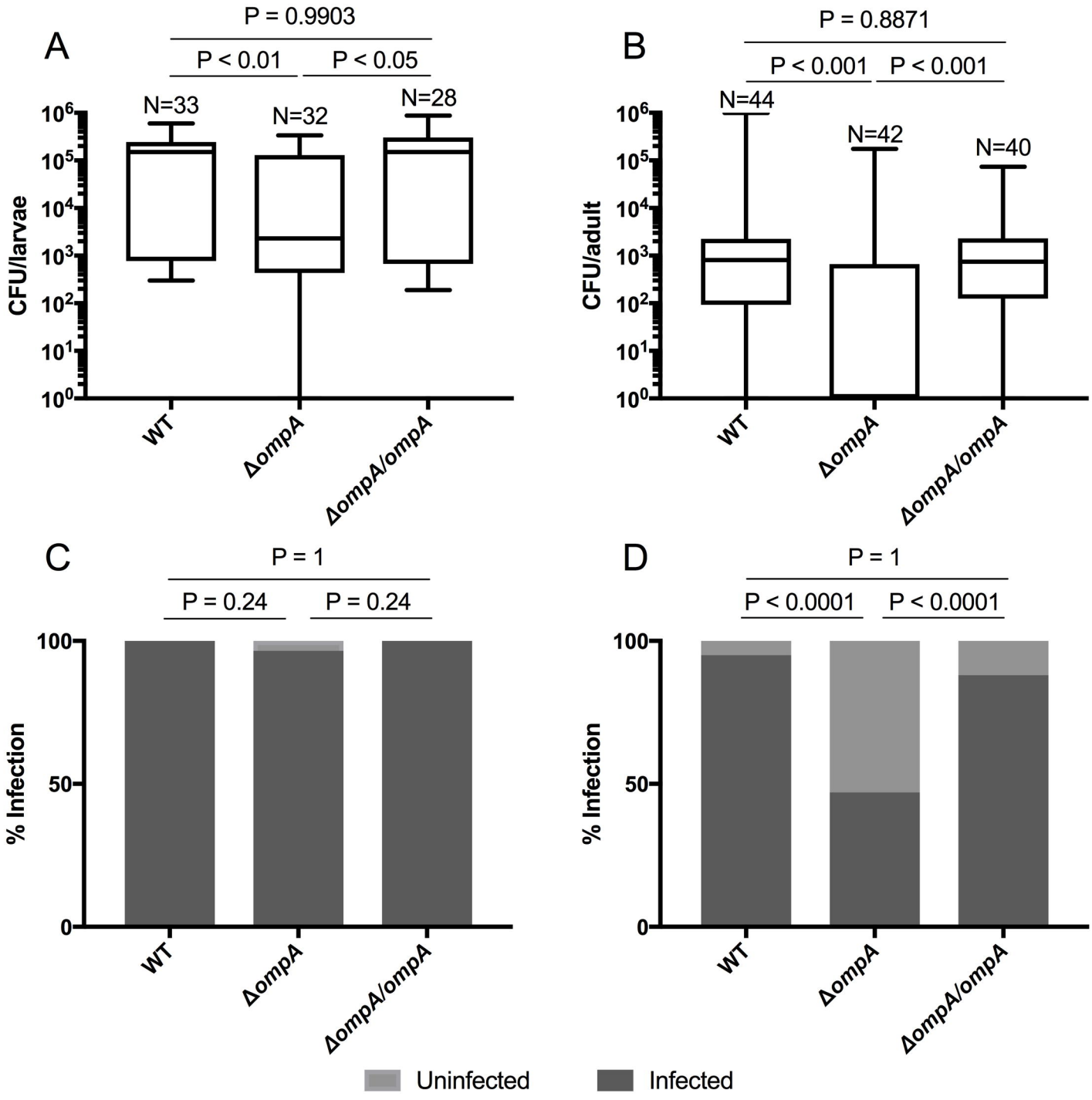
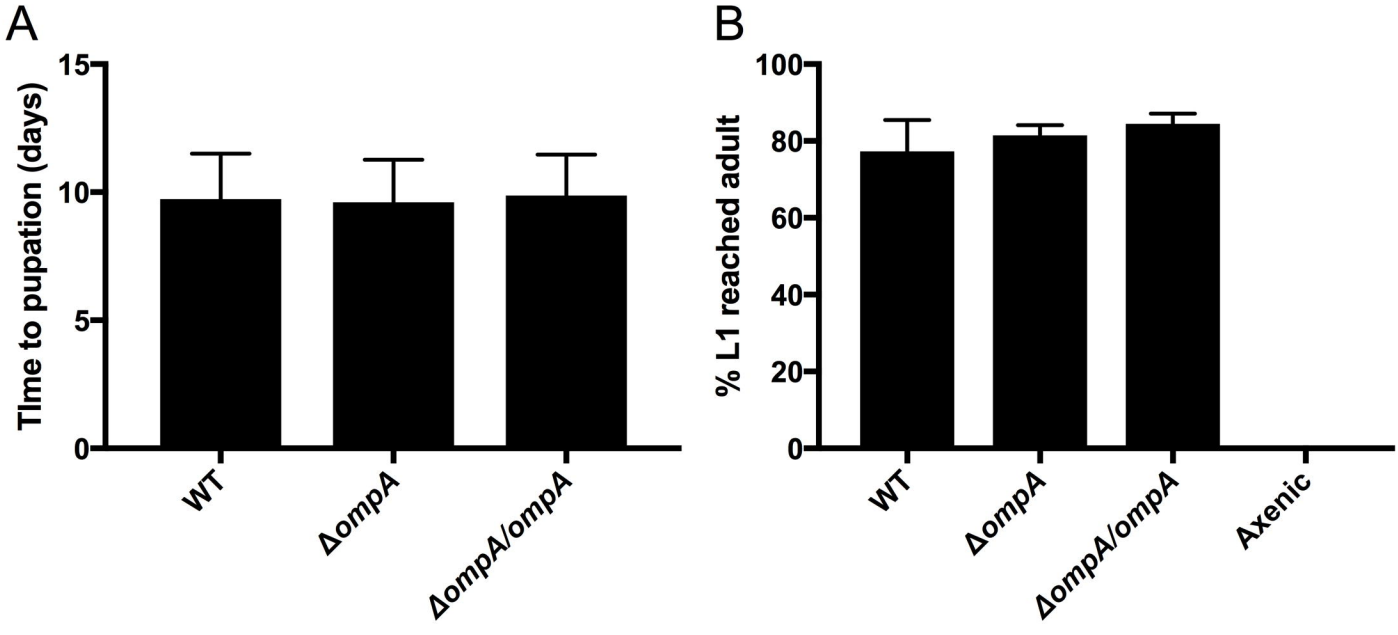
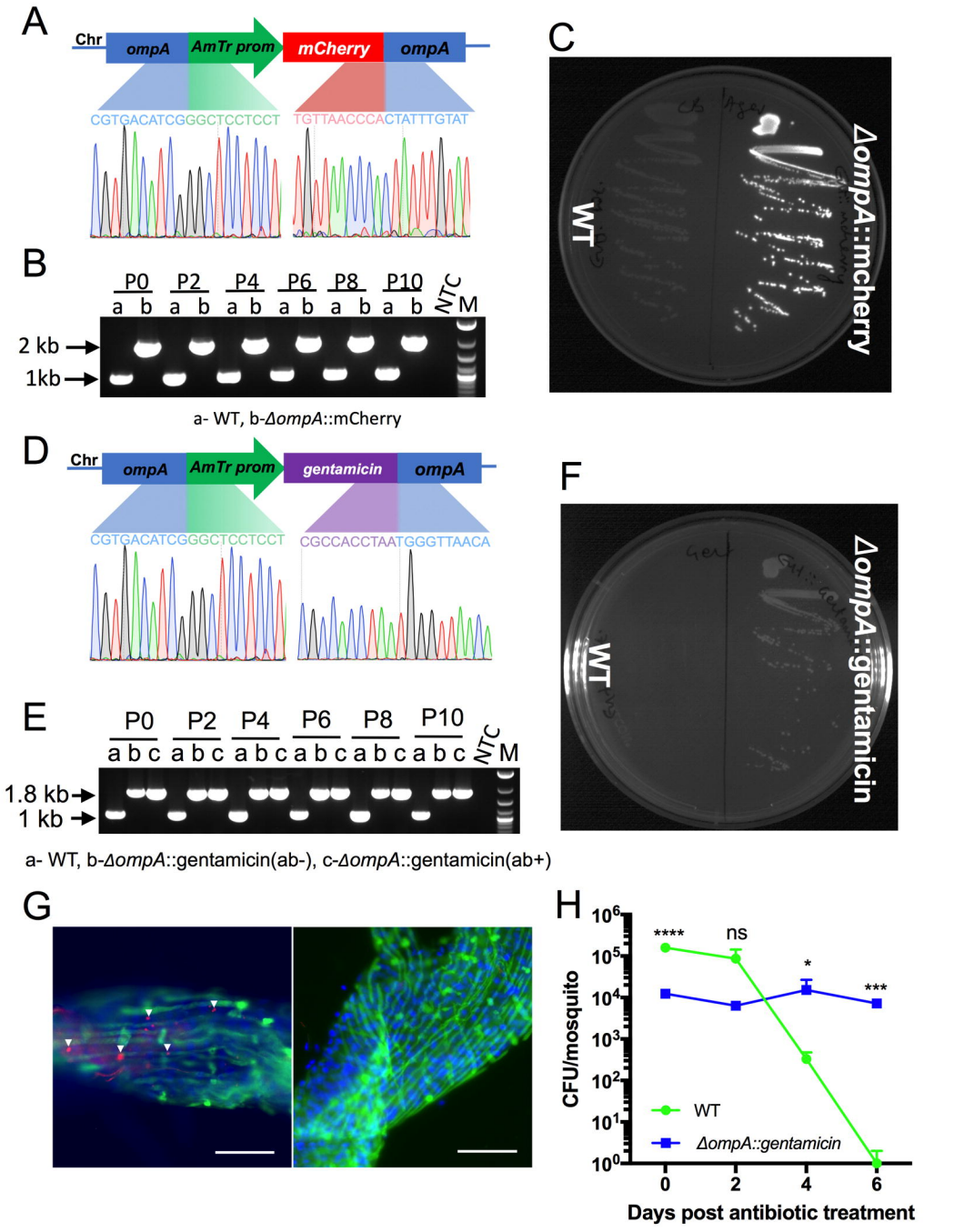
RA
500 bp donor dsDNA
Chr
ΔompA
WT ~1 Kb
ΔompA ~500 bp
C
1001 bp
GATTGCAGTG....AAAGACGTTG
Chr
Ec. ompA
CAGCTATCGCTAACTCAGCC
598 bp
AAATGGGTTA....AGTACAACCT
Chr
Ent. ompA
GTTGGTTTTGGAAACTGTCT


A
OD 600nm
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
24
Time in hrs
WT
ΔompA
ΔompA/ompA
B
P0
P2
P4
P6
P8
WT
ΔompA
WT
ΔompA
WT
ΔompA
WT
ΔompA
WT
ΔompA
NTC
M
1 Kb
500bp
C
WT
ΔompA
ΔompA/ompA
D
CV OD 570 nm
1.5
1.0
0.5
0.0
ns
p<0.0001
p<0.0001
WT
ΔompA
ΔompA/ompA


A
P = 0.9903
P < 0.01
P < 0.05
N=33
N=32
N=28
CFU/larvae
WT
ΔompA
ΔompA/ompA
B
P = 0.8871
P < 0.001
P < 0.001
N=44
N=42
N=40
CFU/adult
C
P = 1
P = 0.24
P = 0.24
% Infection
D
P = 1
P < 0.0001
P < 0.0001
Uninfected
Infected


A
Time to pupation (days)
15
10
5
0
WT
ΔompA
ΔompA/ompA
B
% L1 reached adult
100
80
60
40
20
0
WT
ΔompA
ΔompA/ompA
Axenic


A
Chr
ompA
AmTr prom
mCherry
ompA
CGTGACATCGGGCTCCTCCT
TGTTAACCCACTATTTGTAT
B
P0 P2 P4 P6 P8 P10
a b a b a b a b a b a b
NTC
M
2 kb
1kb
a- WT, b-ΔompA::mCherry
C
WT
ΔompA::mcherry
D
Chr
ompA
AmTr prom
gentamicin
ompA
CGTGACATCGGGCTCCTCCT
CGCCACCTAATGGGTTAACA
E
P0 P2 P4 P6 P8 P10
a b c a b c a b c a b c a b c a b c
NTC
M
1.8 kb
1 kb
a- WT, b-ΔompA::gentamicin(ab-), c-ΔompA::gentamicin(ab+)
F
WT
ΔompA::gentamicin
G
H
CFU/mosquito
Days post antibiotic treatment
****
ns
*
***
WT
ΔompA::gentamicin