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

CRISPR/Cas9 gene editing is a powerful tool to modify bacterial genomes but it has yet to be applied to insect symbionts. To demonstrate that the CRISPR/Cas9 system can be used to genetically alter host-associated bacteria, we deleted the outer membrane protein A (*ompA*) of an *Enterobacter* symbiont of *Aedes* mosquitoes. The Δ*ompA* mutant had an impaired ability to form biofilms and poorly infected *Ae. aegypti*. In adults, the mutant had a significantly reduced infection prevalence compared to the wild type or complement, while no differences in prevalence were seen in larvae, suggesting bacterial genetic factors are important for adult gut colonization. Integration of genes into the symbiont genome demonstrated this technology can be exploited to develop novel symbiotic control strategies. Our results provide insight into the role of the *ompA* gene in host-microbe interactions and confirms CRISPR/Cas9 technology can be employed for genetic manipulation of non-model gut microbes.

Introduction.

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

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

was found to have lower rates of colonization of the midguts of *Anopheles* mosquitoes[26].

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

106

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

Results

Enterobacter biofilm formation in *Ae. aegypti* guts

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

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

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

144 double stranded DNA template that had regions of homology flanking the gRNA site
145 (250 bp for each arm). Using this approach, we successfully deleted a 1001 bp
146 fragment of the *ompA* gene. Of the colonies we screened, we saw an editing at a
147 frequency of 6.25% (N = 48) (Figure 2A). Given our successful editing in *E. coli*, we
148 employed this technique in the non-model *Enterobacter*. However, we altered our
149 editing procedure to delete a 598 bp fragment from the *Enterobacter ompA* gene. This
150 was done to attain a higher frequency of mutants[66] and accommodate a different PAM
151 site in the *ompA* gene of *Enterobacter*. Using a donor template designed for the
152 *Enterobacter ompA* gene that had similar length flanking homology arms as the
153 previous experiment done in *E. coli*, we obtained mutant knockouts at a rate of 32% (N
154 = 50) (Figure 2B). For both bacterial species, Sanger sequencing across the integration
155 site indicated the deletion occurred at the expected loci in the bacterial genome (Figure
156 2C; Supplementary text 1 and 2).

157

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

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

166

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

The role of *ompA* gene in mosquito infection.

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

infection (number of mosquitoes infected) in the larval stage (Figure 4C, Fisher's exact test; WT compared to Δ*ompA* P = 0.24 and Δ*ompA* compared to Δ*ompA/ompA* P = 0.24), in adults, the prevalence of infection was significantly different (Figure 4D, Fisher's exact test; WT compared to Δ*ompA* P < 0.0001 and Δ*ompA* compared to Δ*ompA/ompA* P < 0.0001), with only 45% of adults infected by the Δ*ompA* mutant compared to 95% and 88% by the WT and Δ*ompA/ompA* complement, respectively. We also examined the growth rates of mosquitoes administered with the WT, Δ*ompA* mutant and Δ*ompA/ompA* complement. No significant differences were seen in the time to pupation (Figure 5A) or percentage of first instar larvae that reached adulthood (Figure 5B) between any of the strains.

Integration of genes into the *Enterobacter* chromosome.

We undertook experiments to demonstrate the CRISPR/Cas9 gene-editing approaches can be used to integrate genes into the chromosome of non-model bacteria that associate with mosquitoes. We created two independent transgenic strains that had either, a gene encoding mCherry fluorescence or a gene encoding resistance to the antibiotic gentamicin, inserted into the bacterial chromosome. These genes were integrated into the genome using the same gRNA that was used for deletional mutagenesis, and as such, these insertions also disrupted the *ompA* gene. Sequencing across the integration site indicated the insertion of these genes occurred within the *ompA* gene and thereby disrupted its function (Figure 6A and 6D). Continual subculturing was undertaken for both strains and molecular analysis indicated the stability of these lines for ten generations (Figure 6B and 6E). To demonstrate the

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

229 **Discussion.**

230 We harnessed the CRISPR/Cas9 gene editing system to create knockout mutants in an
231 *Enterobacter* gut symbiont of *Ae. aegypti* mosquitoes enabling us to examine the role of
232 bacterial genetics, specifically the *ompA* gene, in biofilm formation and gut colonization.
233 A deletion of the *ompA* gene of *Enterobacter* decreased bacterial colonization of the
234 mosquito host at both the larval and adult stages after infection in a mono-association.
235 Strikingly, we found this effect was most pronounced in adult mosquitoes with more
236 than half of the mosquitoes not possessing any culturable mutants, while there was no
237 difference in prevalence of infection between the mutant and WT bacteria in larvae. The
238 reduced prevalence of mutant bacteria in adults likely reflects differences in microbial
239 colonization of each mosquito life stage. Larvae are continually subjected to bacteria in
240 the larval water habitat while adults only have a short time frame to acquire bacteria
241 immediately after eclosion, when they are thought to imbibe a small amount of larval
242 water which seeds the gut with microbiota[67]. Our data shows greater variation in
243 colonization of the adult stage between the mutant and WT strains, indicating that the
244 *ompA* gene, and potentially bacterial factors in general, may be critical for colonization
245 of the adult gut. These findings are also consistent with other sequence-based studies,
246 that indicate adult stages have greater variability in species composition of their
247 microbiota, while the microbiome of immature stages reflects the larval water habitat[1-8].
248
249 Overall, our findings are similar to studies done in Tsetse flies whereby an *ompA* mutant
250 of *Sodalis*, an *Enterobacteriaceae* symbiont, has impaired biofilm formation and
251 reduced colonization rates[22,23]. These studies, in conjunction with our work, suggests

252 that the *ompA* gene is imperative for symbiotic associations within dipterans. It also
253 suggests that biofilm formation may be a strategy employed by bacteria to colonize the
254 gut of insects. In pathogenic infections in mammals, biofilms enable bacteria to colonize
255 new niches, promote infection and are associated with virulence[68,69]. Although less is
256 known regarding the importance of biofilm formation in insects, in an artificial
257 *Pseudomonas-Drosophila* infection model, biofilm formation was associated with
258 virulence and host survival[20]. In a natural symbiotic association between bean bugs and
259 *Burkholderia*, disruption of a purine biosynthesis gene in the bacterium also reduce
260 biofilm formation and colonization of the insect[65]. In mosquitoes, gut biofilm formation
261 could also have implications for vector competence as *Chromobacterium*, which was
262 isolated from *Aedes* mosquitoes, produced molecules that inhibited dengue virus only
263 when grown *in vitro* as a biofilm and not when grown in a planktonic state[70]. However, it
264 is unknown if biofilm formation occurred *in vivo*[70]. Our data provide evidence that
265 biofilms occur within the gut of mosquitoes and facilitate host colonization.
266
267 While we have shown that the *ompA* gene of *Enterobacter* is important for host
268 colonization, we see no evidence that deletion of this gene alters mosquito development
269 or growth rates. This is in contrast to the *Riptortus-Burkholderia* symbiosis whereby
270 mutation of the *purT* gene in *Burkholderia* resulted in reduced growth rates and
271 reduction in body weight of the host compared to insects that were infected with the WT
272 bacterium[65]. The difference in our study to the findings in the *Riptortus-Burkholderia*
273 symbiosis could be related to different requirements of the bean bug compared to the
274 mosquito host as well as the different genes mutated in the symbionts. Our findings are

consistent with another study in mosquitoes whereby an *ompA* mutant of *E. coli* was reported to not influence growth when a genomic mutant library was screened in *Ae. aegypti* in a mono-association[71]. Using a similar gnotobiotic system that exploits the ability to sterilize mosquito eggs and rescue development of the insect by supplementation, several recent reports describe approaches to create bacteria-free mosquitoes[62,63]. Here, we reared mosquitoes in a mono-association where they were only subjected to *Enterobacter.* However, more than half the adult mosquitoes inoculated with the Δ*ompA* mutant were not infected by bacteria, but nevertheless, had similar development and growth rates compared to mosquito possessing WT bacteria. It would be intriguing to determine if these uninfected mosquitoes were bacteria-free. If so, the use of mutant bacteria that rescue development but cannot colonize mosquitoes may provide a simple means to create sterile bacteria-free mosquitoes.

CRISPR/Cas9 gene editing has revolutionized genetic approaches in model bacteria[32-35], and while the use of this approach is expanding to other non-model bacteria[37-42], to our knowledge, there are no examples of editing undertaken in symbiotic microbes. Here we demonstrate that editing approaches functional in *E. coli* can be applied to phylogenetically related symbiotic bacteria that associate within the guts of mosquitoes. Our overall goal was to delete the *ompA* gene in *Enterobacter* and therefore we altered our editing strategy when carrying out experiments in the non-model *Enterobacter* compared to our initial attempts in *E. coli*, and as such it is difficult to compare rates of editing between bacterial species. Nevertheless, the number of mutant colonies was considerably greater in *Enterobacter* compared to *E. coli* indicating the editing approach

298 is applicable to this symbiotic *Enterobacteriaceae* bacterium. Similar to our findings, a
299 two-plasmid CRISPR/Cas9 system, exploiting the use of λ-Red recombineering,
300 developed in *E. coli* was also functional in the *Enterobacteriaceae, Taumella citrea*,
301 without the need for modification[36]. Taken together, these findings suggest that
302 CRISPR/Cas9-based approaches may be applicable to a wide range of
303 *Enterobacteriaceae.* Similar to the original study in *E. coli*[35], we observed
304 counterselection escapees in our editing experiments. The no-SCAR approach uses a
305 counterselectoin method to edit bacteria and cells that possess both the Cas9 and the
306 gRNA should not be viable as a result of double stranded breaks in their genome. As
307 the genetic basis of counterselection escape remains unclear and our primary objective
308 was to create a mutant strain, we did not pursue the mechanism behind these escapees
309 further.
310
311 Previous integration attempts in *E. coli* using the no-SCAR approach inserted a small
312 fragment (80 bp) into the bacterial genome[35]. It was hypothesized however, that this
313 approach could be used to integrate larger sized fragments over 1 kb into the genome
314 based on the efficiency of integration and counterselection escape rates, although
315 screening would be required[35]. Here we demonstrate this is indeed feasible as we
316 inserted 1.3 kb and 1.5 kb fragments into the *Enterobacter* genome, and importantly,
317 these genes, driven by the AmTr promoter[72], were functional both *in vitro* and *in vivo*.
318 Our work expands the list of bacteria amenable to CRISPR/Cas9 based genome editing
319 approaches and provides an elegant tool to investigate specific bacterial genes that
320 influence host-microbe interactions in mosquito vectors.

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

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

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

Materials and Methods

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

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

either PCR amplification or by gene synthesis (Genewiz). Regardless of the method of construction, each donor had flanking regions of 250 bp homologous to the target DNA. The resulting colonies were screened for mutations by colony PCR with primers flanking the integration site and positive clones were Sanger sequenced. Positive colonies were grown in LB broth and genomic DNA was isolated. For further validation, the flanking regions of deletion or insertions were amplified and the PCR product Sanger sequenced.

Stability of insertion. The stability of the knockout Δ*ompA* mutant and the knockin *ompA*::gentamicin and *ompA*::mCherry strains was assessed in LB medium. The *omp*A::mCherry and knockout Δ*ompA* mutant cultures were grown for 10 passages in LB broth. At each passage 40 µl of culture was transferred into 4ml fresh LB medium. The *ompA*::gentamicin strain was grown with or without gentamicin (50 µg/mL). Genomic DNA was isolated from the 0, 2, 4, 6, 8 and 10^{th} subculture and PCR that amplified across the integration site was performed.

Complementation of *ompA* mutant. Functional rescue of the *omp*A mutation was achieved by complementing the mutant with the WT gene. The WT *omp*A gene was amplified from *Enterobacter* genomic DNA and cloned into the pRAM-mCherry vector[7] and thereby creating pRAM-mCherry-*Ent-OmpA.* The Sanger sequence-verified plasmid was transformed into the Δ*ompA* mutant, thereby generating the Δ*ompA/ompA* complement strain. Colonies that acquired the plasmid were selected on LB plates containing kanamycin (50 µg/mL).

395

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

406

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

418 then homogenized in 500 µl of 1X PBS and 50 µl of homogenate was plated on LB agar
419 containing 50 µg/mL kanamycin. Similarly, adult mosquitoes were collected 3-4 days
420 post emergence and bacterial infection was quantified in the same manner as larvae. In
421 order to assess the growth of the mosquitoes, time to pupation and growth rate were
422 observed. Time to pupation was determined by quantifying the number of pupae each
423 day post hatching, while survival to adulthood was calculated by quantifying the number
424 of L1 larvae that reached adulthood. The experiment was repeated three times.
425
426 Knock-in mutants were administered to adult *Ae. aegypti* in a sugar meal. Three to four
427 day old mosquitoes were fed with $1x10^7$ of WT or the Δ*ompA*::gentamicin strain for
428 three days in 10% sucrose solution. After three days, mosquitoes were either
429 administered sugar supplemented with gentamicin (50 µg/mL) or sugar without
430 antibiotic. CFUs were determined at days 0, 2, 4, and 6 dpi by plating homogenized
431 mosquitoes (N=10) on LB agar. Similarly, the Δ*ompA*::mCherry and WT *Enterobacter*
432 were fed to mosquitoes and midguts were dissected to assess the colonization of
433 bacteria in the tissue. For visualization of bacteria, midguts were fixed in 1%
434 paraformaldehyde (PFA) in 1X PBS for 30 minutes and permeabilized with 0.01% Triton
435 X-100 in 1X PBS for 20 min. The tissues were stained with 1:250 diluted Phalloidin
436 (Sigma) for 20 minutes and samples were washed twice with 1X PBS for 10 minutes.
437 Finally, midguts were then stained with 1:500 diluted DAPI (Invitrogen) for 10 min.
438 Samples were transferred to slides and mounted with ProLong™ Gold Antifade
439 (Invitrogen). The slides were observed under Revolve FL (ECHOLAB).
440

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Competing interests.

The authors declare no competing interests.

455 **Figure legends.**

456

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

463

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

475

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

478 complement in liquid LB media (A). The stability of mutant was evaluated *in vitro* by
479 continuous subculturing in LB medium (B). Genomic DNA of alternative subcultures was
480 used as template for PCR using gene specific primers that amplified across the
481 deletion. Two separate gel images were merged to create the figure 2B. Passage 8 was
482 run on a separate gel to passages 0 – 6. Biofilm formation was assessed using the CV
483 biofilm assay for the WT, Δ*ompA* mutant and the Δ*ompA/ompA* complement (C).
484 Quantification of the relative biofilm formation normalized by the number of bacteria per
485 well (D).
486
487 **Figure 4. The Δ*ompA* mutant poorly infected mosquitoes.** Infection of *Enterobacter*
488 strains (WT, Δ*ompA* mutant and Δ*ompA/ompA* complement) reared in a mono-
489 association using a gnotobiotic rearing approach for larvae (A) and adults (B). L4 and 3-
490 4 days post emergence adults were screened for bacterial load by plating on LB media
491 to quantify the bacteria. The prevalence of infection (number of mosquitoes infected)
492 between the treatments was calculated comparing number of infected to uninfected
493 larvae (C) or adults (D).
494
495 **Figure 5. The Δ*ompA* mutant does not affect growth rates or development of**
496 **mosquitoes.** The growth rate (time to pupation) (A) and development (percentage of L1
497 larvae to reach adulthood) (B) was observed in mosquitoes infected with *Enterobacter*
498 strains (WT, Δ*ompA* mutant and Δ*ompA/ompA* complement) reared in a mono-
499 association.
500

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

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520

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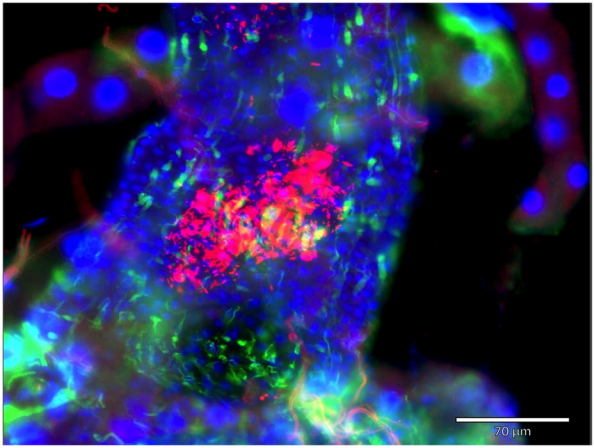
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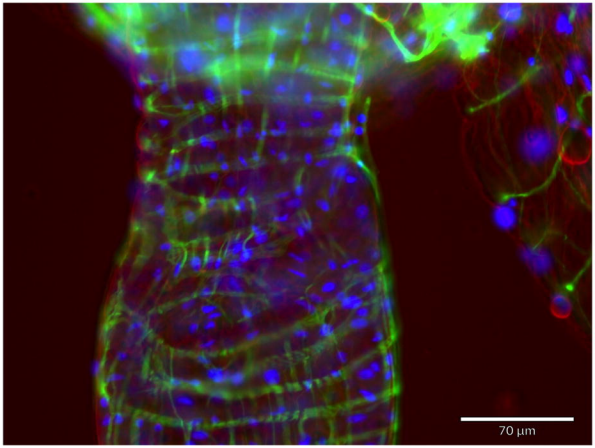
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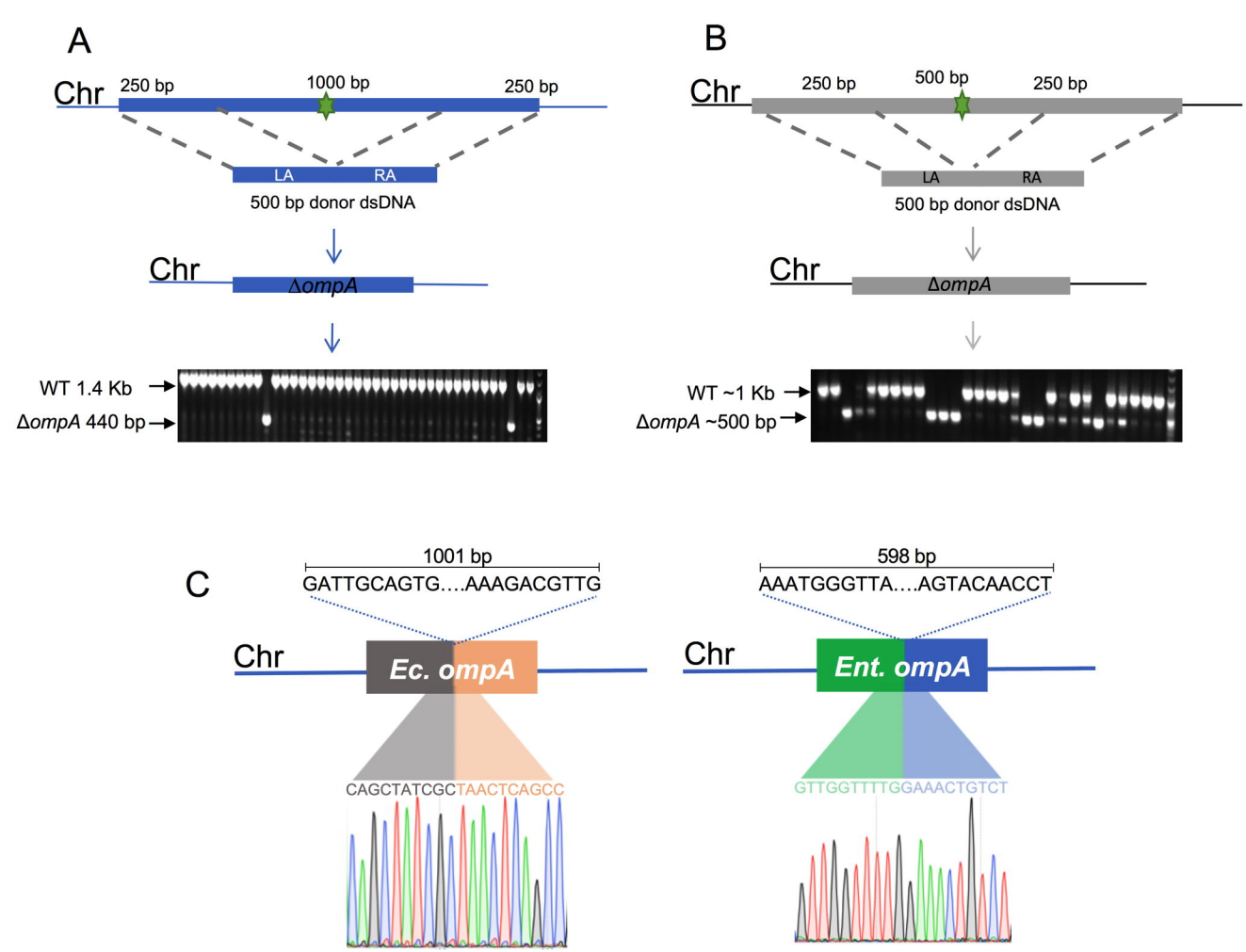
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Chr
250 bp
1000 bp
250 bp
LA
RA
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Chr
ΔompA
WT 1.4 Kb
ΔompA 440 bp
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250 bp
500 bp
250 bp
LA
RA
500 bp donor dsDNA
Chr
ΔompA
WT ~1 Kb
ΔompA ~500 bp
C
1001 bp
GATTGCAGTG….AAAGACGTTG
Chr
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598 bp
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Ent. ompA
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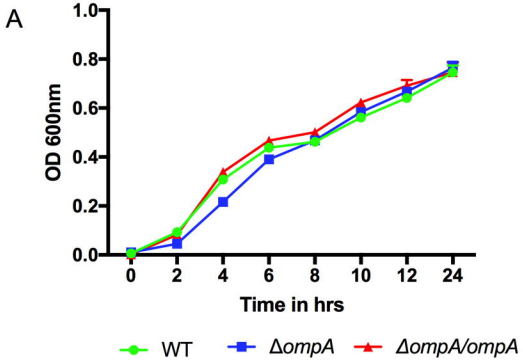
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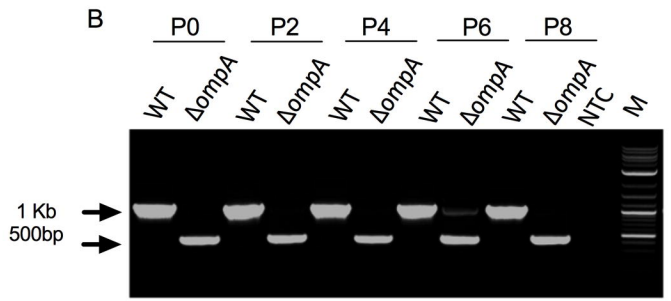
OD 600nm
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
24
Time in hrs
WT
ΔompA
ΔompA/ompA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ΔompA
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M
1 Kb
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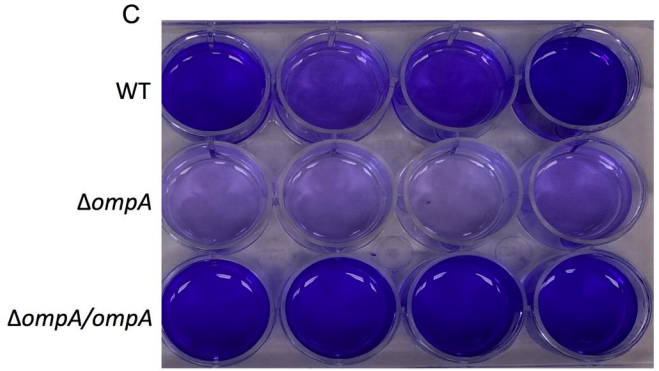
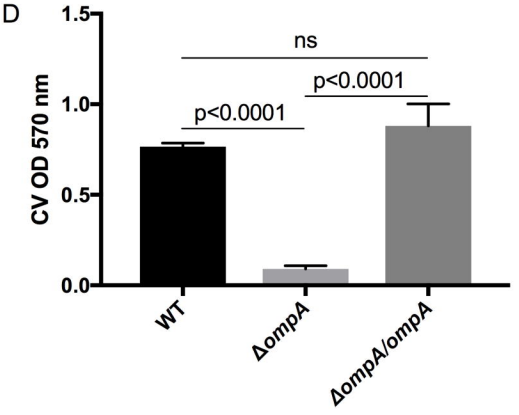
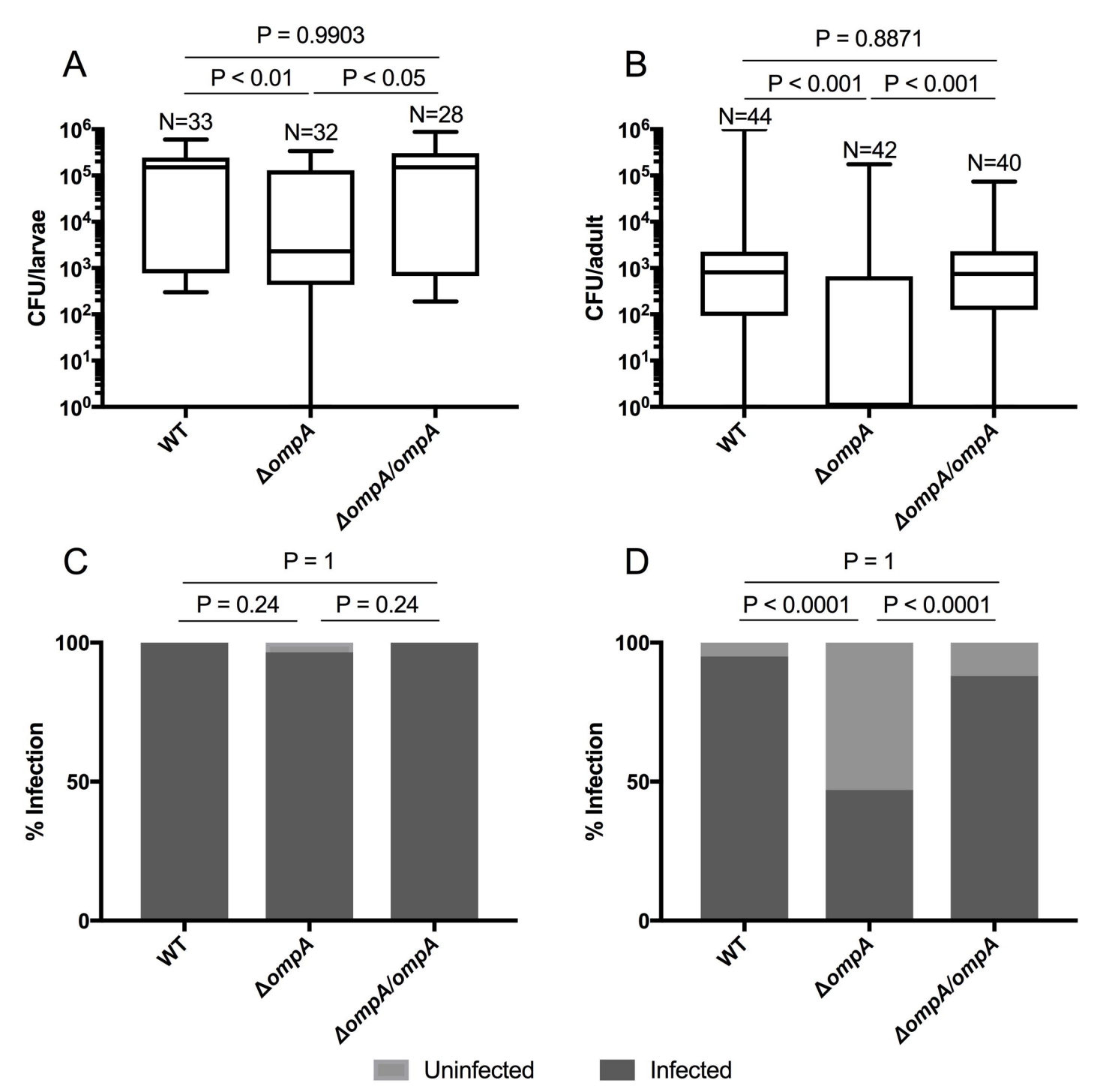
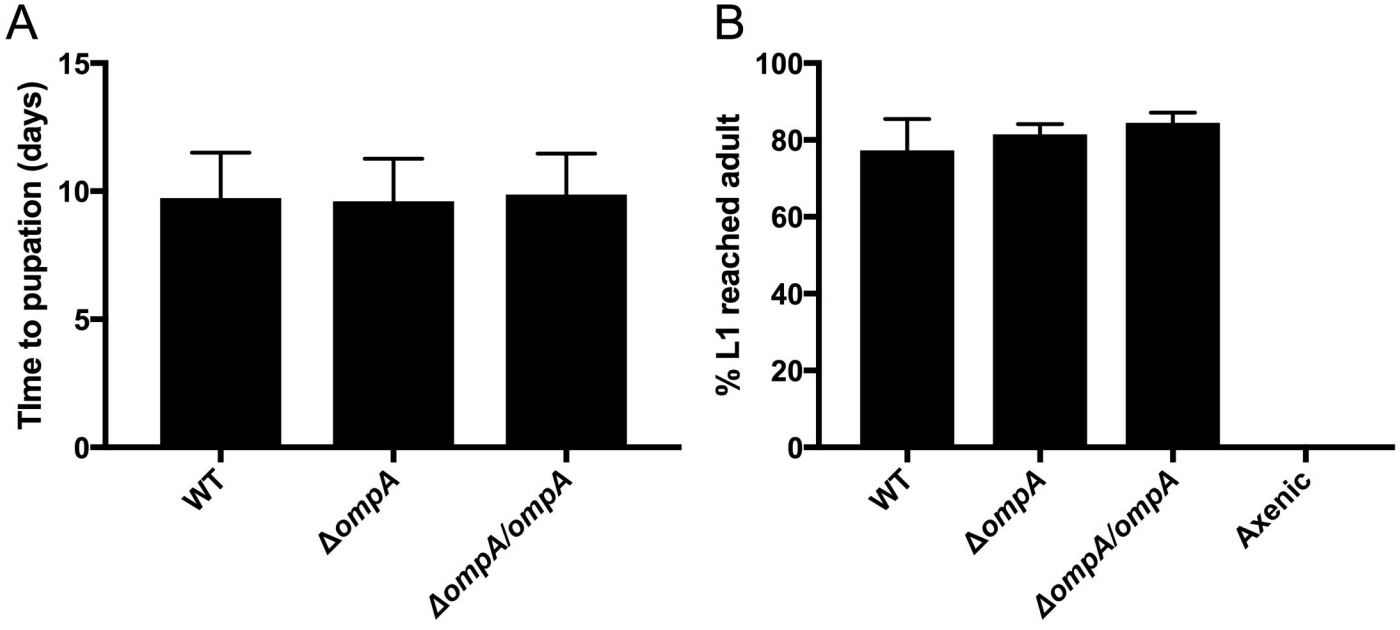
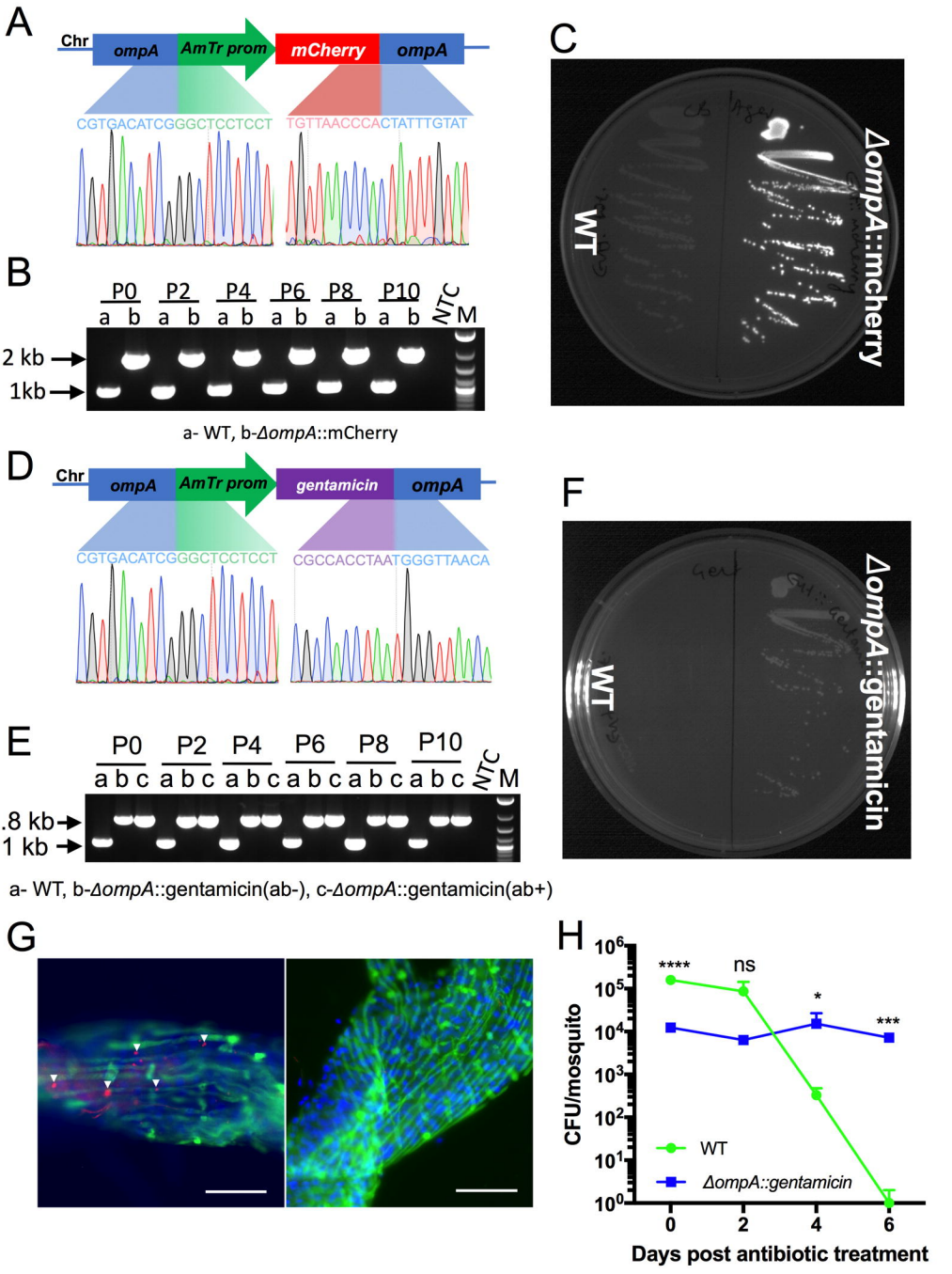

C
WT
ΔompA
ΔompA/ompA


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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ompA
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mCherry
ompA
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B
P0 P2 P4 P6 P8 P10 NTC M
a b a b a b a b a b a b
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 NTC M
a b c a b c a b c a b c a b c a b c
1.8 kb
1 kb
a- WT, b-ΔompA::gentamicin(ab-), c-ΔompA::gentamicin(ab+)
F
WT
ΔompA::gentamicin
G
H
CFU/mosquito
****
ns
*
***
WT
ΔompA::gentamicin
Days post antibiotic treatment