RhlR, but not RhlI, allows P. aeruginosa *bacteria to evade* Drosophila *Tep4-mediated opsonization*

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ABSTRACT

When *Drosophila* flies feed on *Pseudomonas aeruginosa* PA14, some bacteria cross the intestinal barrier and start proliferating intensely inside the hemocoel. This process is limited by hemocytes through phagocytosis. We have previously shown that the PA14 quorum-sensing regulator RhlR is required for the bacteria to elude the cellular immune response. RhlI synthesizes the auto-inducer signal that activates RhlR. Here, we have carefully compared the null mutant phenotypes of *rhlR* and *rhlI*. Surprisingly, unlike *ΔrhlR* mutants*, ΔrhlI* mutants are only modestly attenuated for virulence and are poorly phagocytosed and opsonized in a Thioester-containing Protein4-dependent manner. Likewise, Δ*rhlI* but not Δ*rhlR* mutants colonize the digestive tract of *Caenorhabdtis elegans* and kill it as efficiently as wild-type PA14. Thus, RhlR has an RhlI-independent function in eluding detection or counter-acting the action of the immune system. In contrast to the intestinal infection model, *Tep4* mutant flies are more resistant to PA14 in a septic injury model, also an *rhlR*-dependent property. Thus, the Tep4 putative opsonin can either be protective or detrimental to host defense depending on the infection route.

INTRODUCTION

Drosophila melanogaster is a powerful genetic model organism for the study of innate immunity that has been intensely investigated during the past 25 years (Buchon, Silverman et al., 2014). It thus represents an informative system in which to study host-pathogen interactions using either systemic infection or so-called "natural" infection paradigms, such as oral infection (Bier & Guichard, 2012, Igboin, Griffen et al., 2012, Limmer, Quintin et al., 2011b). Genetic analysis has allowed the detailed dissection of the *Drosophila* systemic immune response to microbial infections (Lemaitre & Hoffmann, 2007). In addition to

52 melanization which is mediated by the protease-mediated cleavage of prophenol oxidase into
53 active phenol oxidase, two major NF-kappaB pathways, Toll and Immune deficiency (IMD),
54 regulate the induction of the expression of genes that encode potent antimicrobial peptides,
55 which are active against most bacteria and fungi (Ferrandon, Imler et al., 2007, Ganesan,
56 Aggarwal et al., 2010, Lemaitre & Hoffmann, 2007). The *Drosophila* systemic immune
57 response is so effective, especially in the case of Gram-negative bacterial infections, that a
58 second arm of host defense, the cellular immune response, has remained comparatively less
59 well studied (Pean & Dionne, 2014). Indeed, blocking cellular immunity through saturation of
60 the phagocytic apparatus with inert particles does not yield a strong susceptibility phenotype
61 of flies infected by *Escherichia coli*, unless the systemic immune response is at least also
62 partially impaired (Elrod-Erickson, Mishra et al., 2000). Nevertheless, we have found that
63 when two opportunistic pathogens *Serratia marcescens* and *Pseudomonas aeruginosa* are fed
64 to *Drosophila*, the cellular immune response plays a key role in controlling the bacteria that
65 escape from the digestive tract (Limmer, Haller et al., 2011a, Nehme, Liegeois et al., 2007).
66 In both cases, the putative phagocytic receptor Eater plays a crucial role and prevents the
67 development of a rapid bacteremia (Kocks, Cho et al., 2005, Limmer et al., 2011a).

68 The thioester-containing protein Tep1 opsonizes bacteria in the mosquito species *Anopheles*
69 *gambiae* (Levashina, Moita et al., 2001). It is unknown whether opsonization also plays a role
70 *in vivo* in *Drosophila*, even though its genome encodes five functional *Tep* loci and a
71 pseudogene (*Tep5*) (Bou Aoun, Hetru et al., 2011). The thioester motif is not present in Tep6
72 and is therefore thought to be nonfunctional. Indeed, Tep6 is required for the establishment of
73 septate junctions in specific parts of the gut, which explains the lethal phenotype of *Tep6* null
74 mutants (Batz, Forster et al., 2014, Hall, Bone et al., 2014). Tep6 has also been shown to
75 induce autophagy in macrophages via a non cell-autonomous process that involves epithelial
76 cells in which it is expressed (Lin, Rodrigues et al., 2017). A previous study failed to find a

77 role of the remaining *Tep* genes (*Tep1* to *Tep4*) in host defense in several models of bacterial
78 or fungal systemic infections (Bou Aoun et al., 2011), although a study reported that *Tep3*
79 mutant flies are highly susceptible the nematode parasite *Heterorhabditis bacteriophora*
80 (Arefin, Kucerova et al., 2014). Interestingly, a study led in *Drosophila* cultured S2 cells
81 reported that *Tep2* is required for the phagocytosis of the Gram-negative species *Escherichia*
82 *coli*, *Tep3* for the uptake of the Gram-positive *Staphylococcus aureus*, and unexpectedly that
83 *Tep6* is required to phagocytose the dimorphic yeast *Candida albicans* (Stroschein-Stevenson,
84 Foley et al., 2006).

85 In contrast to *S. marcescens*, the *P. aeruginosa* strain PA14 ultimately manages to establish
86 an exponential infection in the hemocoel four to five days after its ingestion. In a previous
87 study, we showed that a member of the LuxR family of signal receptor-transcriptional
88 regulators in PA14, RhlR, is required to circumvent the cellular immune response (Limmer et
89 al., 2011a). Indeed, *rhlR* mutants are almost avirulent in an intestinal infection model since
90 they remain at very low levels in the hemolymph and kill the infected flies at a much reduced
91 rate. Interestingly, the cellular immune response remains functional until late stages of a
92 PA14 infection, suggesting that hemocytes are not directly targeted by the PA14 strain, unlike
93 what happens with *P. aeruginosa* strain CHA, which neutralizes *Drosophila* phagocytosis
94 through the secretion of its ExoS toxin into hemocytes (Avet-Rochex, Bergeret et al., 2005).

95 RhlR is the major regulator of one of the three known quorum-sensing systems in *P.*
96 *aeruginosa*. Quorum-sensing systems play a major role in coordinating the expression of
97 virulence genes in several infection models (Coggan & Wolfgang, 2012, Jimenez, Koch et al.,
98 2012, Schuster, Sexton et al., 2013, Williams & Camara, 2009). However, we have failed to
99 uncover a strong role for the other two *P. aeruginosa* quorum sensing systems regulated by
100 LasR and MvfR in the *Drosophila* intestinal infection model (Limmer et al., 2011a). This
101 observation was somewhat unexpected since the Las system appears to function upstream of

102 the Rhl quorum sensing system. RhlR is activated by binding to an auto-inducer molecule,
103 butanoyl-homoserine lactone (C4-HSL), which is synthesized by the RhlI enzyme. The
104 transcription of the *rhlI* and *rhlR* genes is in turn activated by the Las transcriptional regulator
105 LasR (Coggan & Wolfgang, 2012, Jimenez et al., 2012, Schuster et al., 2013, Williams &
106 Camara, 2009). Activation of RhlR takes place when a threshold concentration of C4-HSL is
107 reached, which correlates in *in vivo* studies with a threshold bacterial concentrations reached
108 during exponential growth.

109 Here, we found that the virulence phenotype exhibited by ingested PA14 *rhlI* mutants is
110 strikingly distinct from that exhibited by *rhlR* mutants, suggesting that RhlR can function
111 independently of activation by C4-HSL. We further establish that RhlR, but not RhlI, is
112 required to elude opsonization by a *Tep4*-dependent process. Finally, we establish that in
113 contrast to its protective role during PA14 intestinal infections, Tep4 plays an opposite role in
114 a systemic infection model, possibly by preventing the activation of the phenol oxidase
115 cascade.

116 **RESULTS**

117 ***Δ*rhlI *is more virulent than Δ*rhlR *in the* Drosophila *intestinal infection model***

118 RhlR is activated by the C4-HSL synthesized by the RhlI enzyme, at least in numerous *in*
119 *vitro* studies (Gambello, Kaye et al., 1993, Latifi, Winson et al., 1995, Pesci, Pearson et al.,
120 1997, Seed, Passador et al., 1995). Thus, we expected that *rhlI* and *rhlR* mutants would
121 display the same phenotype. Unexpectedly, however, although a *ΔrhlI* deletion mutant strain
122 was indeed less virulent than wild-type PA14 when ingested by flies, it was significantly
123 more virulent than *ΔrhlR* (Fig. 1A, B). Moreover, whereas the *ΔrhlR* mutants were cleared
124 from the hemolymph, the *ΔrhlI* mutant proliferated in this compartment, although it appeared
125 to grow less rapidly than wild-type PA14 (Fig. 1C, D). Consistent with this latter result, *ΔrhlI*

126 but not *ΔrhlR* triggered the systemic immune response, as monitored by measuring the
127 expression of the *Diptericin* gene (Fig. S1E-F). Similar results were obtained with
128 independent *ΔrhlR* and *ΔrhlI* in frame deletion mutants constructed by another laboratory (Fig.
129 S1) (Hoyland-Kroghsbo, Paczkowski et al., 2017), thereby confirming the correlation
130 between the *ΔrhlR* and *ΔrhlI* null genotypes and the differing *ΔrhlR* and *ΔrhlI* phenotypes.

131 We next tested the survival rates of flies in which the cellular response had been ablated by
132 injecting latex beads (LXB) after feeding on wild-type or *ΔrhlR* or *ΔrhlI* mutant bacteria.
133 Both *ΔrhlR* and *ΔrhlI* killed latex bead-injected flies much faster than PBS-injected control
134 flies, at approximately the same rate (Fig. 1E, F). In addition, both *ΔrhlR* and *ΔrhlI* killed
135 latex bead-injected flies slower than wild-type PA14, with the difference between *ΔrhlI* and
136 wild-type PA14 at the borderline of statistical significance (p=0.07). It is important to
137 determine whether this apparently enhanced virulence of *ΔrhllR* and *ΔrhlI* mutants observed
138 in phagocytosis-impaired flies is just a reflection of the increased virulence of wild-type PA14
139 in these immuno-deficient flies, or whether the enhanced virulence of the *ΔrhlR* and *ΔrhlI*
140 mutants is indicative of the fact that the RhlR-mediated regulatory systems plays an important
141 role in counteracting the cellular immune response. To this end, it is useful to measure the
142 difference in LT_{50} values of control *vs*. latex bead-injected flies (LT_{50}[wt-w^{LXB}]) for each
143 mutant and to compare it to that measured for wild-type PA14. *ΔrhlR* did recover virulence
144 with a LT_{50}[wt- w^{LXB}] of 4.7 days, as compared to 2.4 days for wild-type PA14, which
145 corresponds to the level of recovered virulence reported earlier (Fig. 1G) (Limmer et al.,
146 2011a). With a value of 3.5 days, *ΔrhlI* displayed an intermediate LT_{50}[wt- w^{LXB}], although
147 the significance of the difference with wild-type PA14 or *ΔrhlR* could not be assessed as the
148 *ΔrhlI* values were too spread out (Fig. 1G). Indeed, *ΔrhlI* mutants consistently tended to
149 display a more variable survival phenotype (Fig. 1B).

150 Finally, both the *ΔrhlR* and *ΔrhlI* mutant strains yielded similarly shaped survival curves
151 when used to infect flies with an impaired cellular defense. These survival curves were less
152 steep than those obtained with wild-type PA14, as measured by their Hill coefficients (Fig.
153 1H). This suggests that quorum-sensing is somehow involved in shaping the survival curve.
154 This reflects a collective property of flies placed in the same vial, which succumb less
155 synchronously during infections when the *rhl* quorum sensing system is missing.

156 **rhlI *and wild-type PA14, but not* rhlR, *strains colonize the* C. elegans *digestive tract***

157 The nematode *C. elegans* is a well-established model host to study *P. aeruginosa*
158 pathogenesis (Irazoqui, Urbach et al., 2010, Pukkila-Worley & Ausubel, 2012, Tan, Mahajan-
159 Miklos et al., 1999a). The *C. elegans* intestinal infection model shares some key features with
160 the *Drosophila* model, at least during the initial stages of the infection. For instance, in both
161 models, ingested bacteria are exposed in the gut lumen to antimicrobial peptides and to
162 reactive oxygen species generated by the Dual oxidase enzyme. However, in contrast to
163 ingested *P. aeruginosa* in *Drosophila*, PA14 are not known to escape from the gut
164 compartment during the nematode infection. We therefore tested whether the differences that
165 we observed in the virulence of *ΔrhlR* and *ΔrhlI* mutants in the *Drosophila* intestinal infection
166 assay were reflected in a well-established *C. elegans – P. aeruginosa* nematode "slow killing"
167 survival assay (McEwan, Feinbaum et al., 2016, Tan et al., 1999a). Indeed, two independently
168 constructed in frame *rhlR* deletion mutants in the PA14 background were dramatically less
169 virulent than two independently constructed *rhlI* deletion mutants in their ability to kill *C.*
170 *elegans* (Fig. 2A)

171 The primary *C. elegans* immune response occurs in intestinal epithelial cells and because the
172 worms are transparent, host-pathogen interactions can be easily visualized in the intestinal
173 lumen. Thus, in addition to the *C. elegans* survival assay (Fig. 2A), we also used a

174 quantitative assay (Figs. 2B-E; see Materials and Methods) that monitors the accumulation of
175 live bacterial cells in the intestine of the nematodes using PA14, *ΔrhlR,* and *ΔrhlI* expressing
176 GFP to monitor the level of intestinal colonization. Live wild-type *P. aeruginosa* PA14 cells
177 start accumulating in the intestine 24-48 hours post infection. Consistent with the *Drosophila*
178 infection assays described in Fig. 1, two independent Δ*rhlR* mutants in the PA14 background
179 colonized the *C. elegans* intestine at significantly lower levels than two independent Δ*rhlI*
180 mutants. Indeed, in this colonization assay, the Δ*rhlI* mutants were indistinguishable from
181 wild-type PA14, similar to the results in the nematode killing assay. An alternative
182 explanation for these results is that *C. elegans* preferentially feeds on the Δ*rhlI* mutant
183 compared to the Δ*rhlR* mutant and simply overwhelms the immune response with a larger
184 number of ingested cells. However, this possibility was ruled out by monitoring the pumping
185 (feeding) rate of *C. elegans* feeding on wild-type, Δ*rhlR* and Δ*rhlI* mutants. *C. elegans*
186 pumped at the same rate on all three strains (Fig. S2).

187 ***Phagocytosis protects* Drosophila *against invasion of its hemocoel by wild-type PA14***
188 ***during the early phase of the infection***

189 In the *Drosophila* intestinal infection model, flies constantly feed on PA14 present on a filter.
190 A defining feature of this model is that while bacteria are able to rapidly cross the intestinal
191 barrier to penetrate the hemocoel, as had been previously described for *Serratia marcescens*,
192 the PA14 titer in the hemolymph remains low for the first few days of the infection. After this
193 initial incubation period, there is an exponential proliferation of PA14 in the hemocoel, which
194 coincides with the activation of the systemic immune response. Using bacteria expressing
195 different colored fluorescent proteins, we have previously shown that *S. marcescens*
196 continuously crosses the intestinal barrier during the infection. Fig. 3A-B shows that when
197 flies that have been feeding on dsRed-labeled PA14 bacteria for four days were switched to a

198 filter laced with GFP-labeled PA14, the green bacteria progressively replaced the red bacteria
199 both in the gut and hemocoel compartments. We conclude that *P. aeruginosa*, like *S.*
200 *marcescens*, continuously crosses the intestinal barrier during the infection.

201 Next, we asked at what time periods during an infection was phagocytosis important in
202 preventing PA14 growth in the hemolymph. To this end, we saturated the phagocytic
203 apparatus of hemocytes by injecting latex beads into flies at different time points during
204 infection. As expected, blocking phagocytosis one day prior to the infection led to an earlier
205 demise of the PA14-infected flies compared to PBS-injected control flies. Similar results were
206 found when latex beads were injected four hours or one day after infection, although in the
207 latter case the difference was not significant (its value was nevertheless similar to that
208 obtained by injection one day prior to infection at -1 day; Fig. 3C). In contrast to injecting the
209 latex beads one day after infection, the injection of latex beads four or six days after the
210 beginning of the ingestion of wild-type PA14 did not modify the survival rate of flies. That is,
211 the LT_{50} values were similar to those of control (PBS-injected) flies, consistent with the
212 conclusion that starting about four days after infection the cellular immune response no longer
213 plays a major role in limiting a wild-type PA14 infection.

214 In contrast to wild-type PA14, *ΔrhlR* bacteria were kept in check by phagocytosis at least up
215 to day four and to some extent up to six days after infection (Fig. 3D, F). Phagocytosis was
216 also efficient against *ΔrhlI* bacteria for at least four days (Fig. 3E, F). These data suggest that
217 *ΔrhlR* bacteria are constantly kept in check by the cellular immune response when penetrating
218 the hemocoel, whereas wild-type PA14 ultimately escape this immune surveillance. *ΔrhlI*
219 mutant bacteria display an intermediate phenotype, with a somewhat decreased virulence in
220 flies in which phagocytosis was blocked at day 4 that is similar to that observed with *ΔrhlR*
221 (Fig. 3F).

222 A recent study has reported that hemocytes are recruited to the gut after the ingestion of
223 bacteria (Ayyaz, Li et al., 2015). We confirmed this finding in the case of *P. aeruginosa* oral
224 infection, with a significant recruitment observed at four hours after the beginning of the
225 infection with either wild-type PA14 or *Δrhl* mutants (Fig. 3G-H, Fig. S3). While hemocytes
226 remained associated with the midgut for at least three days after the beginning of the ingestion
227 of wild-type PA14 or *ΔrhlR* bacteria, they were not at three days in the case of *ΔrhlI* bacteria
228 (Fig. 3H). While some ingested bacteria could be detected in the hemocytes recruited to the
229 gut, this phenomenon was not reproducible enough to allow reliable quantification.

230 **Drosophila Tep4 *is required for host defense against ingested PA14***

231 Our previous work had shown that flies mutant for the putative phagocytic receptor gene
232 *Eater* are more susceptible to PA14 ingestion and display a phenotype similar to that obtained
233 by latex bead-mediated ablation of the phagocytic capacity of hemocytes (Limmer et al.,
234 2011a). Thioester -containing proteins have been reported to be required for phagocytosis in
235 mosquitoes and also in cultured *Drosophila* S2 cells (Levashina et al., 2001, Stroschein-
236 Stevenson et al., 2006). We therefore tested mutations affecting the *Tep2*, *Tep3*, and/or *Tep4*
237 genes (Bou Aoun et al., 2011). In the case of *Tep1*, since no mutants were available, we tested
238 this gene using an RNAi transgenic line expressed either in hemocytes or in the fat body.
239 However, we did not observe any change in the virulence of ingested PA14 (Fig. S4A, B).
240 *Tep4* and triple *Tep2-Tep3-Tep4* mutants displayed increased susceptibility to PA14 ingestion,
241 in contrast to *Tep3* and double *Tep2-Tep3* mutants that displayed respectively a somewhat
242 decreased or wild-type susceptibility (Fig. 4A, D-E). Of note, uninfected *Tep3* mutants fed on
243 a sucrose solution displayed an enhanced fitness when compared to wild-type or other *Tep*
244 mutant lines (Fig. S4C). We conclude from these data that Tep4, but not other thioester-
245 containing proteins, is required for host defense against ingested PA14.

246 Next, we found that *ΔrhlR* became as virulent as wild-type PA14 when ingested by *Tep4* or
247 *Tep2-Tep3-Tep4* mutants, which was not observed with the *Tep2* and *Tep2-Tep3* mutant
248 strains (Fig. 4B-E). Interestingly, the injection of latex beads in *Tep4* flies only modestly
249 increased the virulence of *rhlR* bacteria when compared to PBS-injected *Tep4* flies (Fig. S4 D,
250 E), suggesting that phagocytosis of PA14 is severely affected in the *Tep4* mutant. *ΔrhlI*
251 bacteria behaved like *ΔrhlR* bacteria when ingested by *Tep4* (Fig. 4C-E), similarly to flies
252 injected with latex beads (Fig. 1E), although *ΔrhlR* recovered virulence to a much larger
253 extent (3.1 days) than *ΔrhlI* (0.9 days) when ingested by *Tep4* flies. Hence, the behavior of
254 *ΔrhlR* mutant PA14 is similar in *eater* and *Tep4* mutant flies, thereby raising the possibility
255 that both fly genes are involved in the same process, in keeping with an unchanged phenotype
256 of *Tep4* when phagocytosis was blocked by the injection of latex beads (Fig. S4D).

257 ***Phagocytosis of PA14 bacteria is impaired in* Tep4 *mutant hemocytes***

258 To quantitatively monitor the uptake of PA14, we used assays that relied on larval hemocytes.
259 First, we injected heat-killed, pHrodo®-labeled bacteria in wild-type or *Tep4* third-instar
260 larvae. The dye becomes fluorescent when placed in an acidic environment such as that
261 encountered in phagosomes. After 45 minutes, the larvae were bled and a phagocytic index
262 was established. Wild-type hemocytes ingested significantly more PA14 or *ΔrhlR* bacteria
263 than *Tep4* hemocytes (Fig. 5A). There were however no significant differences between heat-
264 killed wild-type PA14 and *rhlR* bacteria uptake by wild-type hemocytes on the one hand, or
265 *Tep4* hemocytes on the other (Fig. 5A). This was not necessarily unexpected as heat-killing
266 likely inactivates *rhlR*-dependent virulence factors and might also alter the surface of bacteria.
267 We therefore modified the assay with live bacteria and used an antibody we had raised against
268 PA14 to immuno-stain the bacteria differentially, prior and after permeabilization of the fixed
269 cells. As before, *Tep4* hemocytes exhibited a decreased uptake of bacteria compared to wild-

270 type hemocytes. However, in the case of live bacteria, *rhlR* bacteria were significantly better
271 phagocytosed than wild-type PA14 bacteria when injected into wild-type or *Tep4* larvae. *rhlI*
272 exhibited an intermediate phenotype in this assay and was not significantly different from
273 either wild-type or *rhlR* bacteria. We conclude that this assay is not sensitive enough to
274 discriminate between these two bacterial mutant strains. We obtained similar results using the
275 *ΔrhlR* and *ΔrhlI* strains generated by another laboratory (Fig. S1G).

276 ***Tep4 opsonizes* rhlR *better than* rhlI *or wild-type bacteria***

277 We next designed an experiment to assess whether Tep4 functions as an opsonin, *i.e.*, that it is
278 deposited on the surface of bacteria to facilitate its detection and subsequent ingestion by
279 hemocytes. To this end, we collected the hemolymph from either wild-type or *Tep4* larvae
280 and incubated it with live bacteria. These bacteria were then retrieved and injected into either
281 naive wild-type or *Tep4* mutant larvae prior to bleeding these injected larvae to be able to
282 count the ingested bacteria as described above (Fig. 6A). Wild-type PA14 were poorly
283 phagocytosed in this assay (medians of phagocytic index lower than 10, Fig. 6C), when the
284 opsonized bacteria were first incubated in the hemolymph of Tep4-containing wild-type
285 larvae and then secondarily injected into wild-type or *Tep4* larvae (although the former
286 yielded a significantly increased phagocytic index, as compared to bacteria incubated first in
287 *Tep4* hemolymph; Fig. 6B-E). In contrast, bacteria that had been first incubated in *Tep4*
288 mutant hemolymph were hardly ingested when re-injected into *Tep4* larvae (Fig. 6E). Re-
289 injection of these bacteria into wild-type larvae recipients modestly increased the phagocytic
290 index (Fig. 6E), which was nevertheless lower than that of bacteria that had been pre-
291 incubated with wild-type hemolymph (Fig. 6C). When these experiments were performed
292 using *ΔrhlR* bacteria that were ultimately injected in *Tep4* larval recipients, it made a major
293 difference as to whether these mutant bacteria had first been pre-exposed to wild-type or *Tep4*
294 hemolymph. The Tep4-dependent opsonization led to a massive uptake of bacteria (median

295 phagocytic value of 162), whereas nonopsonized bacteria (pre-incubated with *Tep4* mutant
296 hemolymph) were hardly ingested (median phagocytic value of 12; Fig. 6D-E). As expected,
297 nonopsonized bacteria that were then injected in wild-type recipients were much better
298 phagocytosed (median phagocytic value of 214), presumably because Tep4 was circulating in
299 the wild-type hemolymph (Fig. 6B). They were nevertheless ingested less efficiently than
300 opsonized bacteria (median phagocytic value of 376; Fig. 6B). Finally, *ΔrhlI* bacteria were
301 also opsonized by Tep4, but significantly less than *ΔrhlR* bacteria (Fig. 6B, D). Again, they
302 displayed a phenotype that was intermediate to that of wild-type PA14 on the one hand, and
303 *ΔrhlR* on the other. We conclude that PA14 and, to a lesser extent, *ΔrhlI* bacteria are less
304 efficiently opsonized and subsequently phagocytosed than *ΔrhlR* bacteria, which are therefore
305 unable to elude the cellular immune response.

306 ***Tep4 plays an adverse role in a PA14 systemic infection model in* Drosophila**

307 A recent study has reported that *Tep4* mutants are more resistant to an infection with the
308 entomopathogenic bacterium *Photorhabdus luminescens* in a septic injury model (Shokal &
309 Eleftherianos, 2017). By injecting several doses of PA14, from 10 to 1000 cfus, directly into
310 the thorax of flies, we also consistently found that *Tep4* mutants were surviving better than
311 wild-type flies in this systemic infection model (Fig. 7A). This difference in survival between
312 *Tep4* and wild-type flies was largely attenuated when *ΔrhlR* bacteria were injected thereby
313 establishing again a relationship of altered virulence of these bacteria in this *Drosophila*
314 genetic background (Fig. 7B). Using the steady-state expression of the antibacterial peptide
315 gene *Diptericin* as a read-out of the activation of the IMD pathway that regulates the systemic
316 immune response against Gram-negative bacteria, we found no difference of expression
317 between wild-type and *Tep4* for doses of 100 or 1000 cfus (Fig. 7C). A difference was noted
318 however at time points 2 and 8h after a challenge with 10 cfus. As a higher induction of the

319 IMD pathway is unlikely to account for the increased resistance of *Tep4* mutants against
320 higher PA14 infectious doses, we tested whether the phenol oxidase cascade might be more
321 efficiently activated in this mutant background, as previously reported (Shokal &
322 Eleftherianos, 2017). Indeed, we found that pro-phenol oxidase was cleaved to some extent in
323 *Tep4* but not in wild-type flies. These data further suggest that PA14 bacteria do not
324 efficiently trigger the prophenol oxidase cascade.

325 **Discussion**

326 In this study, we have analyzed the interactions of *P. aeruginosa* with *Drosophila* from the
327 dual perspective of both pathogen and host. Our data lead us to propose a model in which
328 RhlR plays a pivotal role in virulence by diminishing the ability of the cellular immune arm of
329 the host defense response to detect *P. aeruginosa* once the bacteria have reached the internal
330 body cavity of the insect after crossing the intestinal barrier. Surprisingly, RhlR function in
331 eluding opsonization by Tep4 is at least partially independent of the C4-HSL producing
332 enzyme RhlI. These results as well as those of another study (Bonnie Bassler, personal
333 communication) show that RhlR can function independently of C4-HSL, but they do not
334 formally establish that RhlR is functioning independently from a quorum-sensing system in
335 this function.

336

337 ***A* rhlI-*independent function of* rhlR**

338 *P. aeruginosa* is a pathogen that uses complex signaling mechanisms to adapt to its
339 environment and its three quorum-sensing regulators (LasR, RhlR and MvfR) appear to be
340 involved in the regulation of a variety of virulence-related functions (Coggan & Wolfgang,
341 2012, Jimenez et al., 2012, Schuster et al., 2013). *In vitro* studies, sometimes complemented
342 by *in vivo* experiments, have revealed that these quorum-sensing systems are intricately

343 intertwined. It was thus unexpected that only the *P. aeruginosa* RhlR regulator appears to
344 play a critical role for virulence in the *Drosophila* intestinal infection model and not the two
345 other quorum sensor regulators (Limmer et al., 2011a). Here, we report that *ΔrhlR* null
346 mutants consistently display virulence levels that are much weaker than those observed with
347 *ΔrhlI* mutants (Fig. 1B), do not proliferate in the hemolymph in contrast to *ΔrhlI* mutants, and
348 are phagocytosed and opsonized in a Tep4-dependent process more efficiently than *ΔrhlI* and
349 wild-type bacteria. These observations suggest that RhlR functions at least partially
350 independently from RhlI and presumably independently from C4-HSL activation. In contrast,
351 *ΔrhlI* mutants exhibit an impaired virulence in both wild-type and *Tep4* immuno-deficient
352 flies, similarly to *ΔrhlR* mutants. Both *ΔrhlI* and *ΔrhlR* mutants display survival curves with
353 shallow slopes. Thus, the partially overlapping phenotypes of *ΔrhlI* and *ΔrhlR* in flies with
354 impaired cellular immunity open the possibility that RhlR may also partially function together
355 with RhlI as a classical quorum-sensing regulator in a process that remains to be delineated.
356 Interestingly, it appears that RhlR controls gene expression for biofilm formation both in a
357 C4-HSL-dependent and C4-HSL independent manner (Bonnie Bassler, personal
358 communication). This putative conventional function of RhlR plays however a minor role in
359 virulence of PA14 in *Drosophila*, as mirrored by the weak virulence-related phenotypes of
360 *ΔrhlI* mutants documented in our study.

361 One possibility is that RhlR gets activated, perhaps in a C4-HSL-independent manner, by an
362 as yet unidentified quorum-sensing compound. Of note, RhlR does not appear to be activated
363 by 3-oxo-C12-HSL (Bonnie Bassler, personal communication), the LasR ligand, and, in any
364 case, *lasR* and *lasI* mutant bacteria display at best only a modestly decreased virulence
365 phenotype in the *Drosophila* infection model (Fig. S5). The diketopiperazines (DKPs)
366 represent a candidate family of RhlR-activating compounds (Holden, Ram Chhabra et al.,
367 1999); however, at least one study failed to detect any interaction of these compounds with

368 LuxR proteins (Campbell, Lin et al., 2009). The resolution of this issue will require testing
369 mutants that affect the synthesis of DKPs.

370 Another hypothesis to consider is that RhlR may function independently of auto-inducer
371 molecules. RhlR forms dimers in the presence or absence of C4-HSL (Ledgham, Ventre et al.,
372 2003). Further studies reported that RhlR functions as a repressor when unbound to C4-HSL
373 (Anderson, Zimprich et al., 1999, Medina, Juarez et al., 2003). Interestingly, RhlR dimers
374 seem to bind its target DNA sequence with an altered conformation (Medina et al., 2003).
375 Finally, transcriptomics studies on *lasR-rhlR* double mutants also revealed several target
376 genes that appear to be repressed by either LasR or RhlR (Schuster, Lostroh et al., 2003,
377 Wagner, Bushnell et al., 2003). Thus, a repressor function for RhlR unbound to C4-HSL
378 cannot be excluded at this stage. Of note, a limitation of all these studies is that they were
379 performed *in vitro* and not *in vivo*.

380 Finally, our studies on the inactivation of the cellular immune response at different time
381 points of the infection further support a quorum sensing-independent role of RhlR. Our study
382 revealed that phagocytosis is required only when very few bacteria are present in the
383 hemolymph, that is, during the first days of the infection. A caveat here is that we cannot
384 exclude the possibility that C4-HSL or other cryptic autoinducers might be produced by the
385 bacteria present in large numbers in the gut compartment. However, if autoinducers, including
386 C4-HSL, were produced in the intestinal lumen and able to cross the digestive barrier, it is
387 difficult to understand why they would not immediately activate RhlR resulting in full-blown
388 bacteremia without the observed lag before the exponential proliferation phase in the
389 hemocoel. This hypothesis also does not account for why the *rhlI* virulence phenotype is
390 much weaker than that of *rhlR*, unless this reflects the differing opsonization properties of
391 these mutants.

392 ***RhlR counteracts the cellular host defense by eluding detection by Tep4***

393 Our phagocytosis and opsonization data are consistent with a model in which RhlR controls
394 the expression of gene products that might mask the site being recognized by Tep4 or a TEP4-
395 associated protein, presumably on the cell wall. Alternatively, RhlR may actively inhibit the
396 uptake of opsonized bacteria. The masking or inhibition of ingestion processes may be
397 sensitive to heat, as wild-type heat-killed bacteria appeared to be more efficiently taken up by
398 hemocytes than live ones (compare median values for PA14WT in Fig. 6A to those in 6B).
399 Alternatively, the processes may be unstable and require permanent maintenance that can no
400 longer be achieved when the bacterial cells are killed.

401 Insect thioester-containing proteins belong to the complement family, and have been shown to
402 be involved in the opsonization of bacteria in mosquitoes. In *Drosophila*, Tep2 has been
403 reported to be required for the uptake of *Escherichia coli*, a Gram-negative bacterium, by
404 cultured S2 cells (Stroschein-Stevenson et al., 2006). In contrast, we find no involvement of
405 Tep2 in our *in vivo* intestinal infection model with *P. aeruginosa* but do detect a requirement
406 for Tep4 in phagocytosis and opsonization assays. Given that the structure of mosquito
407 thioester-containing protein 1 is similar to that of complement family C3, a well-described
408 opsonin, our data are compatible with a model of direct opsonization of bacteria by Tep4.

409 ***A host factor plays opposite roles in host defense against the same pathogen according to***
410 ***the infection route***

411 The finding that Tep4 plays a protective function in the intestinal infection model whereas it
412 is detrimental in the case of a direct systemic infection is paradoxical. This may actually
413 represent two faces of the same phenomenon. PA14 may have developed a stealth strategy to
414 avoid detection by the immune system of living organisms and thus may actively hide any
415 features that might reveal its presence. We propose here that RhlR is playing a critical role in

416 a program that renders PA14 furtive, in keeping with our finding that bacteria likely need to
417 be alive to escape phagocytosis efficiently (Fig.5). As a result of RhlR action, only a few sites
418 would be available on the surface of the wild-type bacteria for Tep4 direct or indirect binding.
419 There, Tep4 would mediate opsonization and then subsequent phagocytosis of the bacteria,
420 which is an efficient defense when few bacteria enter the hemocoel after escaping from the
421 digestive tract. Our data in the systemic infection model are compatible with the possibility
422 that Tep4 competes for these sites with Pattern Recognition Receptors (PRRs) that activate
423 the systemic humoral immune response and/or the prophenol oxidase cascade. It is likely that
424 small peptidoglycan (PGN) fragments released by proliferating bacteria represent a major
425 trigger of the IMD pathway in addition to large PGN fragments directly sensed by PGRP-LC,
426 thereby accounting for the apparent normal expression of *Diptericin* when flies are challenged
427 with the higher PA14 inocula. In contrast, we have previously established that some fungi and
428 Gram-positive bacteria trigger the phenol oxidase cascade through defined PRRs (Matskevich,
429 Quintin et al., 2010). The situation is less clear as regards Gram-negative bacteria. While the
430 original characterization of PGRP-LE suggested that it was able to trigger the phenol oxidase
431 activation cascade (Takehana, Katsuyama et al., 2002), with some genetic data suggesting that
432 it may actually act non cell-autonomously (Takehana, Yano et al., 2004), subsequent studies
433 have documented a role for PGRP-LE as an intracellular sensor for PGN fragments (Bosco-
434 Drayon, Poidevin et al., 2012, Ferrandon et al., 2007, Yano, Mita et al., 2008). Thus, further
435 work will be required to identify whether Tep4 actually competes with PRRs in the detection
436 of pathogens. A major challenge will be to establish how RhlR influences the surface
437 properties of PA14.

438 ***A similar or a distinct role for RhlR in bypassing host defenses in* C. elegans?**

439 Except for fungal invasion of the epidermis by nematophagous fungi, how *C. elegans* senses
440 infections remains poorly understood (Zugasti, Bose et al., 2014). The finding that *ΔrhlR*

441 mutants are much less virulent than Δ*rhlI* mutants in a *C. elegans* infection model, and that
442 *ΔrhlR* mutants fail to colonize the intestinal tract of worms might be due either to an impaired
443 escape from detection by the immune system or to a defective resistance to its action. It is not
444 clear, however, that RhlR-mediated regulation of the production of toxic phenazines, as
445 shown in the case of PA14-mediated "fast killing" of *C. elegans* (Bonnie Bassler, personal
446 communication), is the reason the *ΔrhlR* mutant is so dramatically impaired in the *C. elegans*
447 "slow killing" assay used in our study in Fig. 2A. The major difference in the fast and slow *C.*
448 *elegans* killing assay is the composition of the agar medium in which the *P. aeruginosa* is
449 grown and on which the killing assays are performed (Mahajan-Miklos, Tan et al., 1999, Tan
450 et al., 1999a, Tan, Rahme et al., 1999b). Fast killing is mediated by phenazines (Cezairliyan,
451 Vinayavekhin et al., 2013, Mahajan-Miklos et al., 1999) whereas slow killing is multi-
452 factorial (Feinbaum, Urbach et al., 2012), and PA14 mutants deficient in the production of
453 phenazines do not exhibit a significant killing defect in the slow killing assay (Tan et al.,
454 1999b). In contrast to *Drosophila*, no cellular host defense has been detected in *C. elegans*
455 and is unlikely to be involved in the immune response to intestinal infection. The use of *ΔrhlR*
456 mutants will open the way to the identification of the relevant host defense systems that are
457 circumvented by wild-type PA14 bacteria. In any case, the lack of a significant phenotype of
458 the *ΔrhlI* mutants in the *C. elegans* killing and intestinal proliferation assays is striking. This
459 can be exploited in future studies to help elucidate the underlying *rhlI*-independent
460 mechanisms involved in RhlR-mediated regulation of virulence.

461

462 Finally, *ΔrhlR* mutants exhibit reduced dissemination capacities in a rodent lung infection
463 model when compared to *ΔrhlI* or wild-type PA14 (Bonnie Bassler, personal communication).
464 By analogy to our findings in the *Drosophila* intestinal infection model, it will be interesting

465 to determine whether the complement system restricts the systemic escape of *ΔrhlR* mutants
466 from the mouse lung into the periphery.

467 **MATERIALS AND METHODS**

468 Many methods employed in this study have been described in detail in Haller *et al.* (2014).

469 ***C. elegans* killing and intestinal accumulation assays**

470 The "slow-killing" of *C. elegans* by *P. aeruginosa* was monitored using automated image
471 analysis as previously described (McEwan et al., 2016, Stroustrup, Ulmschneider et al.,
472 2013). To monitor the accumulation of *P. aeruginosa* in the *C. elegans* intestine, wild-type
473 (N2) animals were used for all experiments. Worms were reared on non-pathogenic *E. coli*
474 *OP50* on nematode growth media at 25°C. Synchronized L4 worms (fourth larval stage) were
475 transferred to slow kill (SK) nematode growth media agar plates containing a lawn of *P.*
476 *aeruginosa* PA14::GFP strains. Post infection at 24 and 48 hours approximately 20 worms are
477 picked onto a 2% agar pad that contain the paralyzing agent levamisole (1mM). The worms
478 were imaged in the GFP channel using a Zeiss Apotome microscope with the same exposure
479 time for all the worms on wild type PA14 and the *rhlR* and *rhlI* mutants. Post acquisition the
480 images were processed using ImageJ software and the area and fluorescence intensity was
481 measured. The relative fluorescence intensity is plotted and a non-parametric Mann-Whitney
482 test was used to determine statistical significance.

483 **Opsonization assay of live bacteria**

484 Overnight cultures of of PA14, *rhlR*, *RhlI*, *sltB1*, *XcpR*, and *vfR* mutants were concentrated to
485 OD10 in PBS. Twenty third instar larvae were bled in 150 µL of the bacterial preparations
486 and incubated at room temperature for 30 to 45 min. Samples were centrifuged at 500 rcf for
487 15 min and the pellet (containing larval debris) was removed. A second centrifugation was
488 performed at 3500 rcf for 15 min to retrieve bacteria in the pellet, that was re-suspended in
489 10 µL PBS. A5001 or *tep4* third instar larvae were injected with 32.2 nL of the live bacteria

490 solutions, using a Nanoject apparatus (Drummont). After 60-90 min of incubation, one larva
491 was bled in each well of an 8-well pattern microscopy slide that contained PBS. The cells
492 were left to settle to the bottom for 30 min and were then fixed in 4 % paraformaldehyde for
493 15 min, in a humid chamber. The samples were washed twice in PBS and they were stained
494 with a 1/500 diluted rabbit antiserum against PA14 in a PBS solution with 2 % BSA for 2
495 hours at room temperature. The cells were incubated with a FITC-labeled goat anti-rabbit
496 secondary antibody (Invitrogen) in a PBS solution with 2 % BSA for 2 hours at room
497 temperature. After a 20 min permeabilization step in a PBS solution with 0.1 % Triton X-100
498 and 2 % BSA, a second round of staining with a 1/500 diluted rabbit antiserum against PA14
499 in a PBS solution with 0.1 % Triton X-100 and 2 % BSA was performed for 2 hours at room
500 temperature. The samples were then incubated with a Cy3-labeled goat anti-rabbit secondary
501 antibody (Invitrogen) in a PBS solution with 0.1 % Triton X-100 and 2 % BSA for 2 hours at
502 room temperature. The slides were mounted in Vectashield with DAPI (Vector Laboratories)
503 and analyzed using a Zeiss Axioskope 2 fluorescent microscope. 40 to 50 cells per larva were
504 analyzed: the number of red fluorescent bacteria that were not green fluorescent was counted
505 for each DAPI-positive hemocyte, and the phagocytosis index was calculated (% of
506 phagocytes containing at least 1 only-green bacterium)×(mean number of only-green bacteria
507 per positive cell). We used the nonparametric Mann-Whitney test for statistical analysis.
508

509 **Phenol oxidase cleavage assay**

510 The procedure was performed as described (Leclerc, Pelte et al., 2006), except that
511 hemolymph loads were not adjusted by measuring the protein content of the extracted
512 hemolymph. The antibody used has been generated by Dr. H. M. Müller against *Anopheles*
513 phenol oxidases (Muller, Dimopoulos et al., 1999). The ratio of cleaved to noncleaved form
514 was performed by densitometry scanning.

515 **Statistical analysis**

516 All statistical analysis were performed on Graphpad Prism version 5 (Graphpad software Inc.,
517 San Diego, CA). Details are indicated in the legend of each figure.

518

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528

529 **AUTHOR CONTRIBUTIONS**

530 SH, AF, SL, and DF conceived the *Drosophila* experiments and analyzed the data. SH
531 performed the majority of these experiments, with later work performed by AF with an input
532 from SL; SS performed the precursor experiments that led to this work. Work on the septic
533 injury model has been performed by JC, with some help from ZL. *ΔlasR*, ΔlasI, ΔrhlR and
534 *ΔrhlI* that were constructed by ED and SY, except when indicated otherwise. AH and FMA
535 conceived the *C. elegans* experiments and analyzed the data, which were obtained by AH. SH,
536 DF, and FMA wrote the manuscript, with inputs from other co-authors.

537 **CONFLICT OF INTEREST**

538 The authors report no conflict of interests.

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696

697 **FIGURE LEGENDS**

698

699 **Figure 1: *RhlI* displays a distinct phenotype from that of *rhlR* in the *Drosophila***
700 **intestinal infection model.**

701 Survival experiment of wild-type flies (*w*) fed on wild-type PA14 bacteria or Δ*rhlR* and Δ*rhlI*
702 mutants.

703 A. Representative survival curves of infected and noninfected (NI) flies. Flies died faster from
704 the infection with PA14 WT than Δ*rhlR* and Δ*rhlI*. Flies infected with Δ*rhlI* exhibited an
705 intermediate survival phenotype. One representative experiment out of seven is shown.
706 Statistical analysis of the data is shown in panel B.

707 B. Pooled LT_{50} data of wild-type flies (w^{A5001}) following intestinal infections with PA14 WT,
708 Δ*rhlR* or Δ*rhlI*. LT_{50} of flies after infection with PA14 WT was significantly lower than with
709 Δ*rhlR* (***p=0.0003) and ΔrhlI (*p=0.0385). Flies were significantly more susceptible to
710 infection with Δ*rhlI* than with Δ*rhlR* (**p=0.0047). The LT_{50} data from seven survival
711 experiments are displayed (biological duplicates are also shown as there was as much
712 variability between experiments as within experiments), red bars indicates medians. Statistics
713 have been performed using a non-parametric test (Mann Whitney).

714 C-D. Bacterial titer of the hemolymph collected from flies that had ingested wild-type or
715 mutant PA14 as indicated, three (C) and five (D) days after the ingestion of wild-type PA14
716 or mutants as indicated. In this series of experiments, flies infected with PA14 WT had started
717 to succumb by day five and were therefore not analyzed. Statistics have been performed using

718 the Mann Whitney test; medians are shown. p values for C are respectively from left to right:
719 0.003, 0.002, and 0.005. P<0.0001 for D.

720 E. Survival curves of wild-type and latex bead-injected flies after intestinal infection with
721 PA14 bacteria. In latex bead-injected flies Δ*rhlI* regains virulence. Note however that the shift
722 in virulence is of the same magnitude as that observed for wild-type PA14 and contrasts with
723 the large shift observed with Δ*rhlR*.

724 F. Pooled LT_{50} data of latex bead-injected flies (*w*-LXB) survival experiments. *w*-LxB flies
725 died significantly slower after Δ*rhlR* infection than with PA14 WT (**p=0.0065). A slight
726 decrease of virulence, but at the border of significance, was observed between PA14 WT and
727 Δ*rhlI* (p=0.0726). No difference in virulence was detected between Δ*rhlR* and Δ*rhlI*
728 (p=0.3056). Data represent the LT_{50}s from five experiments (biological duplicates are also
729 shown as there was as much variability between experiments as within experiments), red bars
730 indicates medians. Statistics have been performed using a non-parametric test (Mann
731 Whitney).

732 G. Pooled data of the difference of LT_{50}s between *w* and *w*-LXB flies after intestinal infection
733 with either PA14 WT, the Δ*rhlR* mutant or the Δ*rhlI* mutant. There is only a significant
734 difference between PA14 WT and the Δ*rhlR* mutant (*p=0.0244) but not the Δ*rhlI* mutant.
735 Data represent the LT50s from five experiments (biological duplicates are also shown as there
736 was as much variability between experiments as within experiments), red bars indicates
737 medians. Statistics have been performed using the Mann Whitney test.

738 H. Hill coefficients of latex bead-injected flies in PA14 infection. Hill coefficients give an
739 indication on the slope of the survival curves. Survival curves of *w*-LXB flies infected with
740 PA14 wild-type (PA14 WT) had a significant higher Hill coefficient than survival curves of
741 flies infected with Δ*rhlR* (**p=0.0092) or Δ*rhlI* (*p=0.0405). No significant difference in Hill
742 coefficient was detected between survival curves of flies infected with Δ*rhlR* or Δ*rhlI*
743 (p=0.6243). The results from three experiments are shown; red bars indicate medians. Mann
744 Whitney tests were used for all statistical analyses.

745 **Figure 2: *rhlR* mutants are impaired in their ability to kill *C. elegans* and do not**
746 **colonize the *C. elegans* intestine.**

747 A, an automated *C. elegans* life-span machine was used to monitor the survival of worms in a
748 *P. aeruginosa*-mediated killing "slow killing" assay (McEwan et al., 2016). Approximately
749 200 wild-type *C. elegans* nematodes were fed wild-type PA14, *ΔrhlI, or ΔrhlR PA14* mutants

750 (constructed in the Ausubel laboratory (au) or Bassler (bb) laboratories. P<0.001 (log-rank
751 test) for PA14 vs *ΔrhlI,* PA14 vs *ΔrhlR, or ΔrhlI vs ΔrhlR*. The experiment was repeated
752 twice with similar results.

753 B-E, *C. elegans* wild-type N2 animals were fed wild-type PA14, *ΔrhlI, or ΔrhlR PA14*
754 mutants (constructed in the Ausubel laboratory (B and D) or Bassler laboratories (C and E)
755 expressing GFP. At 48 h post infection, 13-18 worms infected with wild-type PA14 or the
756 *ΔrhlI* or *ΔrhlR* mutants were imaged in the green fluorescent channel.

757 B and C. Representative images are shown.

758 D and E. Images were quantified using ImageJ. There was a significant difference in the
759 levels of live bacteria between wild type PA14 and *ΔrhlR* (p = 0.034 (D) and 0.0097 (E)), and
760 there were significant differences between the *ΔrhlR* and *ΔrhlI* mutants (p = 0.0012 (D) and
761 0.0042 (E)), using a non-parametric Mann-Whitney test. The differences between *rhlI* and
762 wild-type PA14 were not significant (p = 0.16 (D) and p = 0.95 (E)). The experiments were
763 repeated at least two times with similar results.

764 **Figure 3: Phagocytosis is required during the early stage of the infection in *Drosophila*.**

765 A and B, Wild-type *Drosophila* were orally infected with wild-type PA14 expressing dsRed
766 (PA14-dsRed). After 4 days infected flies were transferred to tubes containing wild-type
767 PA14 expressing GFP (PA14-GFP). At 5 days of the infection (one day after transferring flies
768 to PA14-GFP), most PA14 bacteria in the *Drosophila* gut expressed dsRed and only 10%
769 expressed GFP (A). However, at 6 days (2 days after the transfer of flies to PA14-GFP), only
770 GFP positive bacteria were detected in the gut. Similarly, in the hemolymph of these flies, a
771 majority of PA14-dsRed were detected at 5 days but at 6 days only PA14-GFP were detected
772 in the hemolymph.

773 C, D and E, Measured LT_{50}s from survival experiments of flies after intestinal infection with
774 wild-type PA14 (C), Δ*rhlR* (D) or Δ*rhlI* (E) mutants and injection of either latex beads (LXB,
775 grey dots) or PBS (black dots) at different time points of the infection. Latex beads or PBS
776 were injected either one day before the infection started (-1d) or four hours (+4h), one day
777 (+1d), four days (+4d) or six days (+6d) after the infection started. White dots correspond to
778 the survival of infected, uninjected flies. (C) LT50s of w^{A5001}-LxB are significantly lower than
779 w^{A5001} only at -1d (**p=0.0086) and +4h (*p=0.0154). (D) LT_{50}s of *w*-LXB flies are
780 significantly lower than *w* at most times during the infection (-1d: ***p=0.0002, +4h:
781 ***p=0.0002 and +4d: **p=0.0069). (D) A similar phenotype is observed with flies infected

782 with Δ*rhlI* (-1d: *p=0.0395, +4h: **p=0.0085, +1d: ***p=0.0002 and +4d: *p=0.0400). Note
783 however that for injections of latex beads at day4 the difference is reduced, as compared to
784 earlier time points of injection of latex-beads. The cumulative LT_{50} data from at least three
785 experiments (only two experiments for Δ*rhlI*) are shown, except for +d6; red bars indicates
786 medians. Statistical analyses were done with an unpaired t-test.

787 F. Difference of LT_{50}s between *w*-PBS and w^{A5001}-LXB flies after intestinal infection with
788 either PA14 WT, the Δ*rhlR* mutant *or the* Δ*rhlI* mutant. Data represent the differences of
789 LT_{50}s (*w*-PBS and w^{-}LxB) from at least two experiments, red bars indicates medians.

790 G. Guts of transgenic flies with GFP-labeled hemocytes were dissected in a manner that
791 preserves the association of hemocytes with the digestive tract and were examined by
792 fluorescence confocal microscopy. Green: GFP; blue: DAPI staining of nuclei. Scale bars:
793 100µM.

794 H. Analysis of hemocytes recruited to the fly intestine upon the infection with either wild-
795 type PA14, or Δ*rhlR* or Δ*rhlI* mutant bacteria. Both wild-type PA14 and the Δ*rhlR* or Δ*rhlI*
796 mutant bacteria induce a recruitment of hemocytes to the gut (4h after the beginning of the
797 infection, for each bacteria p<0.0001). At one day, there are slightly more hemocyte recruited
798 when infected with wild-type PA14 compared to the mutant. While at 3 days after the
799 beginning of the infection there are more hemocytes recruited when infected with the Δ*rhlR*
800 mutant bacteria (p=0.0025 between Δ*rhlR* and Δ*rhlI*). Data represent 3 pooled experiments.
801 Red bars represent the medians.

802

803 **Figure 4: *RhlR* circumvents *Tep4*-mediated host defense**

804 A and B. *Drosophila* wild-type flies (w^{A5001}), single mutants *Tep3* and *Tep4*, double mutant
805 *Tep2,3* and triple mutant *Tep2,3,4* were orally infected with PA14 wild-type (A) or the Δ*rhlR*
806 mutant (B) in parallel experiments.

807 A. *Tep4* and *Tep2,3,4* mutant flies are significantly more susceptible to PA14 infection
808 compared to w^{A5001}. No difference in survival was detected between the *Tep2,3* mutant and
809 w^{A5001}. Surprisingly *Tep3* mutants seemed to be more resistant to infection. B, A strong
810 enhancement of Δ*rhlR* virulence is observed with *Tep4* and *Tep2,3,4* mutants compared to
811 w^{A5001} flies. *Tep2,3* and w^{A5001} exhibited nearly the same rate of death when challenged with
812 Δ*rhlR* . The *Tep3* mutant seemed again to be more resistant to the infection. In A and B, one

813 representative experiment out of three (each with biological triplicates, except for uninfected
814 flies) is shown.

815 C. The survival of *Tep4* flies infected with PA14 WT, Δ*rhlR*, Δ*rhlI* bacteria was examined.
816 Three independent experiments have been performed, each with three biological replicates,
817 and one representative experiment is shown.

818 D. Quantification of the experiments shown in PanelC. The triplicates were assessed as
819 independent experiments as there was as much variability between experiments as within
820 experiments. p values comparing the LT_{50}s of mutants versus PA14 WT are from left to right:
821 0.07, 0.003. (Mann-Whitney test).

822 E. Difference of LT_{50}s between *w* and *Tep4* flies after intestinal infection with either PA14
823 WT, Δ*rhlR*, or Δ*rhlI* bacteria. p values comparing the LT_{50}s of mutants versus PA14 WT :
824 *** : $p<0.0001$ (Mann-Whitney test).

825 **Figure 5: Tep4 is required for phagocytosis of *rhlR* mutant bacteria**

826 A. Heat-killed pHrodo®-labeled bacteria of the indicated genotype were injected into either
827 wild-type or *Tep4* third instar larvae and incubated for 45 minutes. The hemocytes were then
828 retrieved. Bacteria present in phagosomes were fluorescent and used to measure the
829 phagocytic index. Bacteria injected into *Tep4* were significantly less phagocytosed than those
830 injected into wild-type larvae: $p<0.0001$ for PA14 WT and $p=0.04$ for Δ*rhlR* (Mann-Whitney
831 test). Medians are shown.

832 B. The experiment is similar to that shown in A, except that live bacteria were used and a
833 differential antibody staining protein was used to reveal phagocytosed bacteria. PA14 WT,
834 Δ*rhlR*, Δ*rhlI* bacteria were injected in wild-type or *Tep4* larvae. Δ*rhlR* bacteria were more
835 readily phagocytosed than PA14 WT both by wild-type ($p=0.01$) or *Tep4* ($p=0.04$) hemocytes
836 (Mann-Whitney test). Medians are shown.

837

838 **Figure 6: Tep4 is an opsonin that preferentially detects *rhlR* over *rhlI* mutant bacteria.**

839 A. Scheme of the experimental procedure. Live bacteria were incubated with either wild-type
840 or *Tep4* hemolymph and were thereafter injected into naive larvae, which were either wild-
841 type or *Tep4*. The phagocytosis index was then measured as in Fig. 6B.

842 B-E. Bacteria pre-incubated with wild-type or *Tep4* hemolymph are represented in pairs,
843 respectively with filled (left) and open (right) circles. Medians are shown. Data were analyzed
844 using the Mann-Whitney test. ***: $p<0.0001$

845 B. PA14 WT, Δ*rhlR*, and Δ*rhlI* bacteria were injected into wild-type larvae. Stars above
846 symbols report the statistical significance of the data when compared to PA14 WT. **:
847 $p=0.007$

848 D. PA14 WT, Δ*rhlR*, and Δ*rhlI* bacteria were injected into *Tep4* larvae. **: $p=0.01$

849 C, E. These panels display respectively a magnification of B and D to show the low
850 phagocytic index associated with PA14 WT uptake.

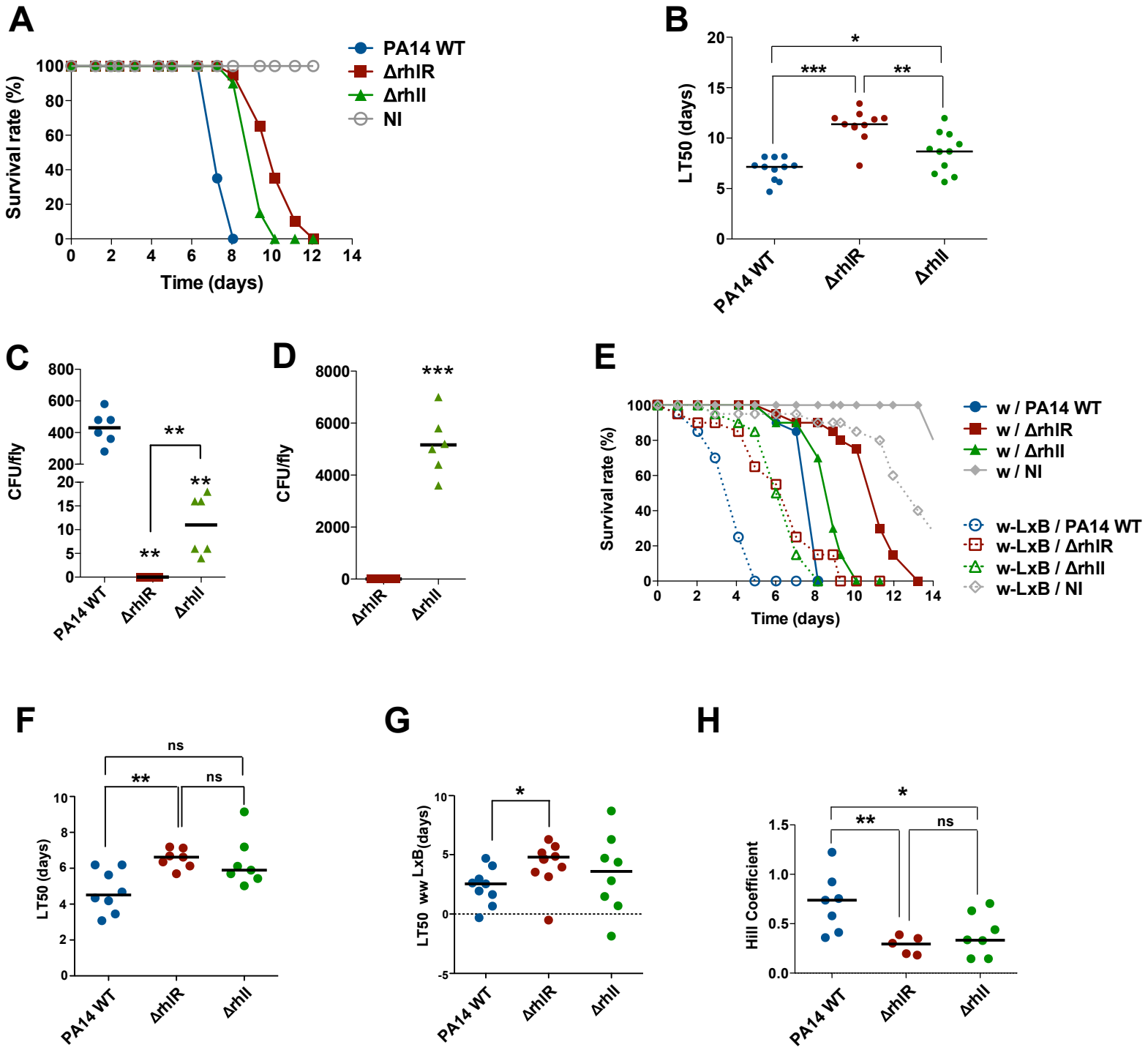
851

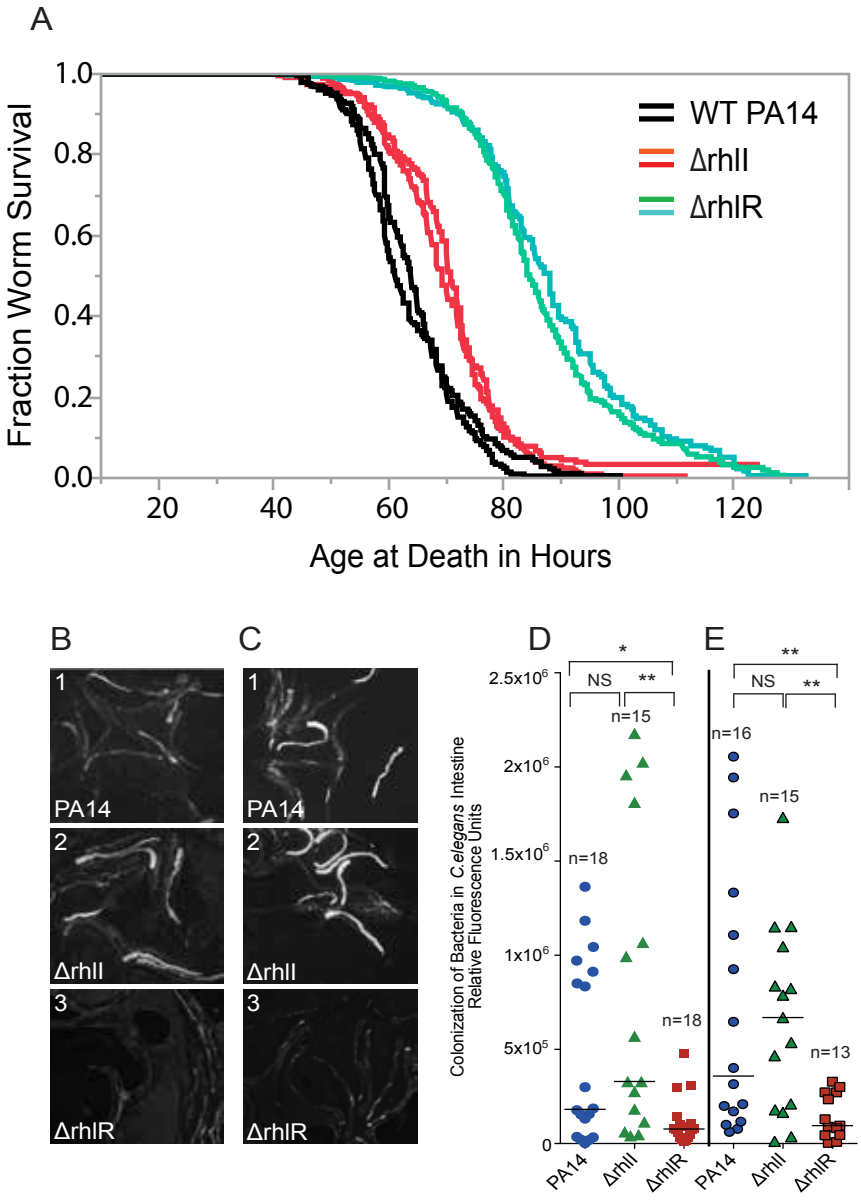
852 **Figure 7: *Tep4* mutants are more resistant to a systemic infection with PA14**

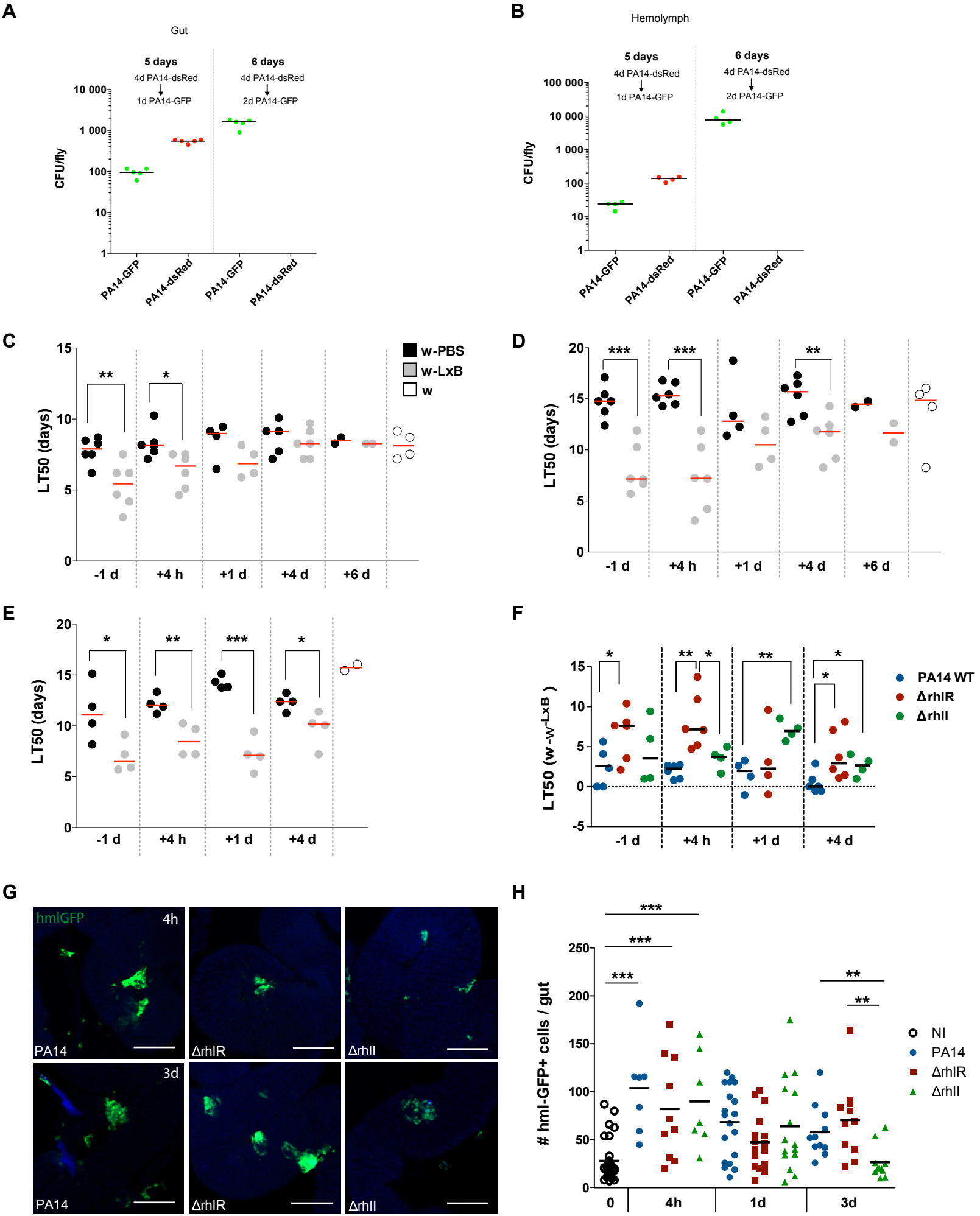
853 A and B. The survival of wild-type and *Tep4* flies was examined, after injection of PBS as a
854 non-infected control (NI), or 10, 100, 1000 cfus of PA14 WT (A) or PA14 Δ*rhlR* (B) bacteria.

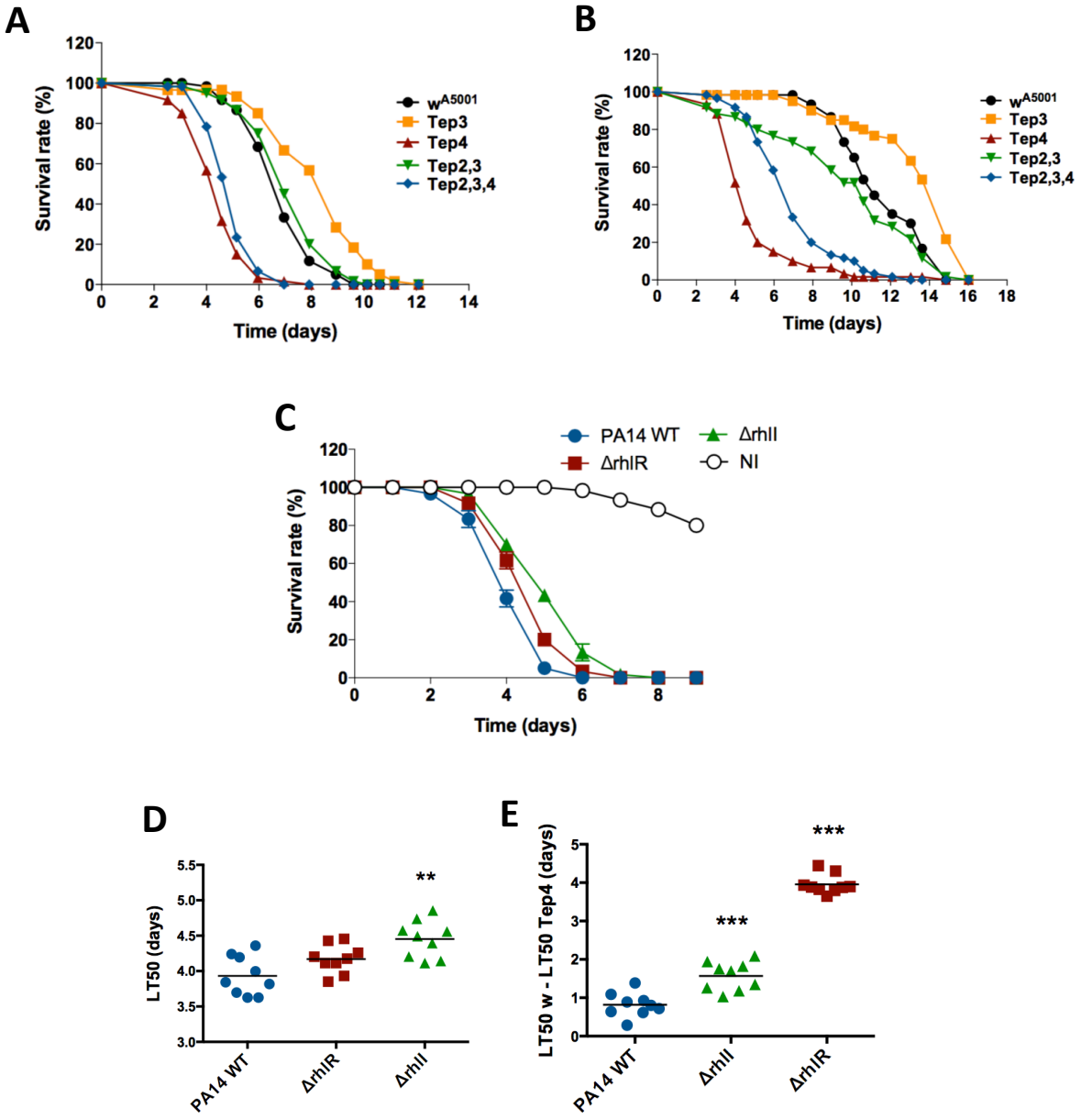
855 C. Relative *Diptericin* expression was measured by RT-qPCR in wild-type and *Tep4* flies, 2,
856 8 or 24h after injection of PBS (NI), or 10, 100, 1000 cfus of PA14 WT bacteria. Each
857 experiment was performed independently three times and a representative experiment is
858 shown (A-C).

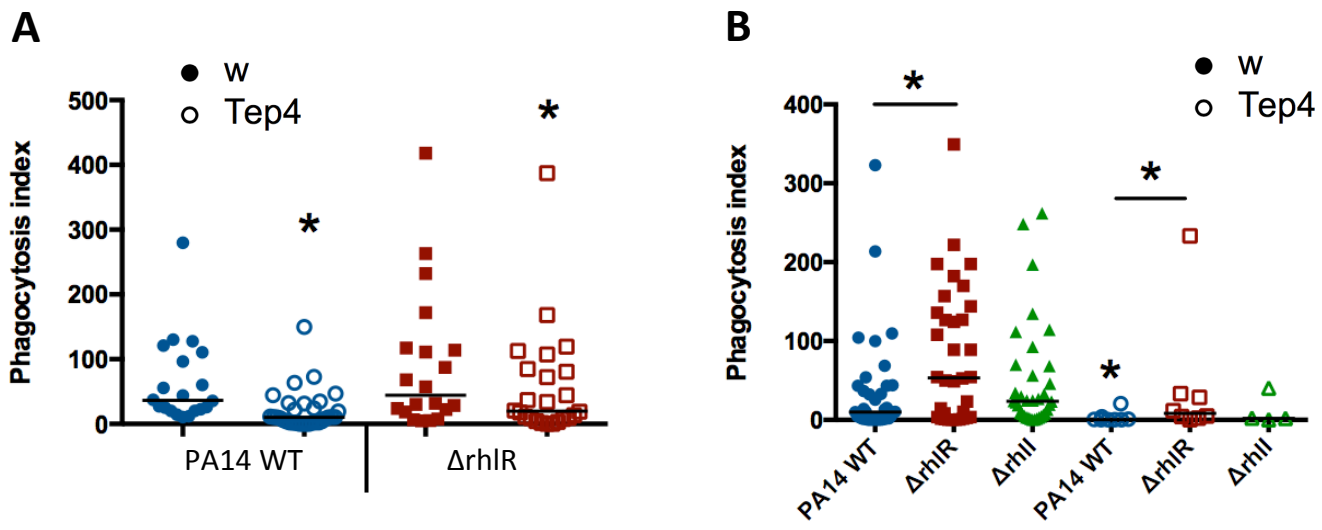
859 D. The cleavage of the prophenol oxidase have been analyzed 2 hours after injection of 1000
860 cfus of PA14 WT bacteria or PBS, by Western-blotting using a pan-phenol oxidase antibody.
861 The intensity of the prophenol oxidase (PPO) and of the cleaved active phenol oxidase
862 (activated PO) bands was measured and the ratio of the measurements is shown below the
863 blot.

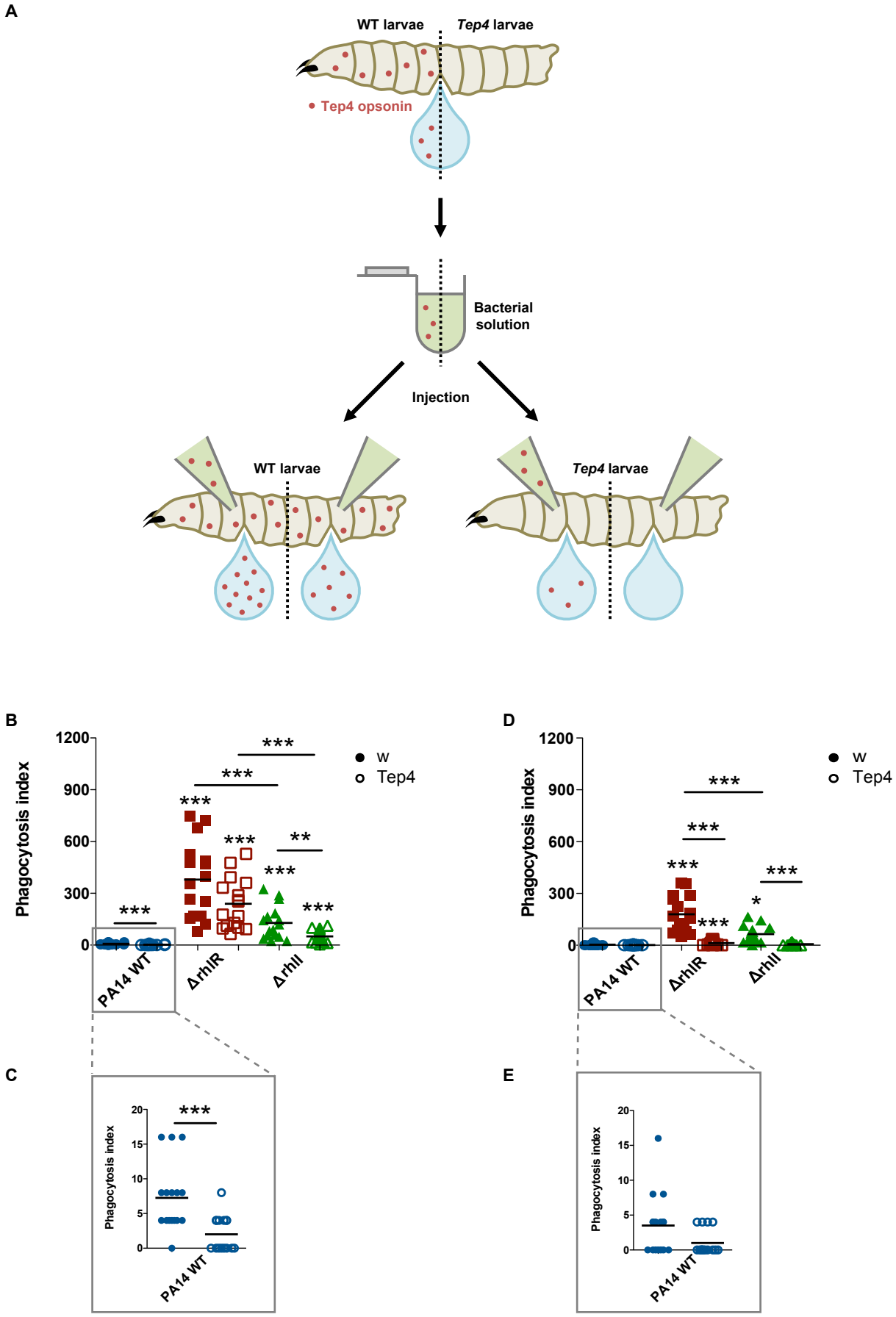

A
Survival rate (%)
Time (days)
PA14 WT
ΔrhlR
ΔrhlI
NI
B
LT50 (days)
PA14 WT
ΔrhlR
ΔrhlI
C
CFU/fly
PA14 WT
ΔrhlR
ΔrhlI
D
CFU/fly
ΔrhlR
ΔrhlI
E
Survival rate (%)
Time (days)
w / PA14 WT
w / ΔrhlR
w / ΔrhlI
w / NI
w-LxB / PA14 WT
w-LxB / ΔrhlR
w-LxB / ΔrhlI
w-LxB / NI
F
LT50 (days)
PA14 WT
ΔrhlR
ΔrhlI
G
LT50 w-w LxB (days)
PA14 WT
ΔrhlR
ΔrhlI
H
Hill Coefficient
PA14 WT
ΔrhlR
ΔrhlI

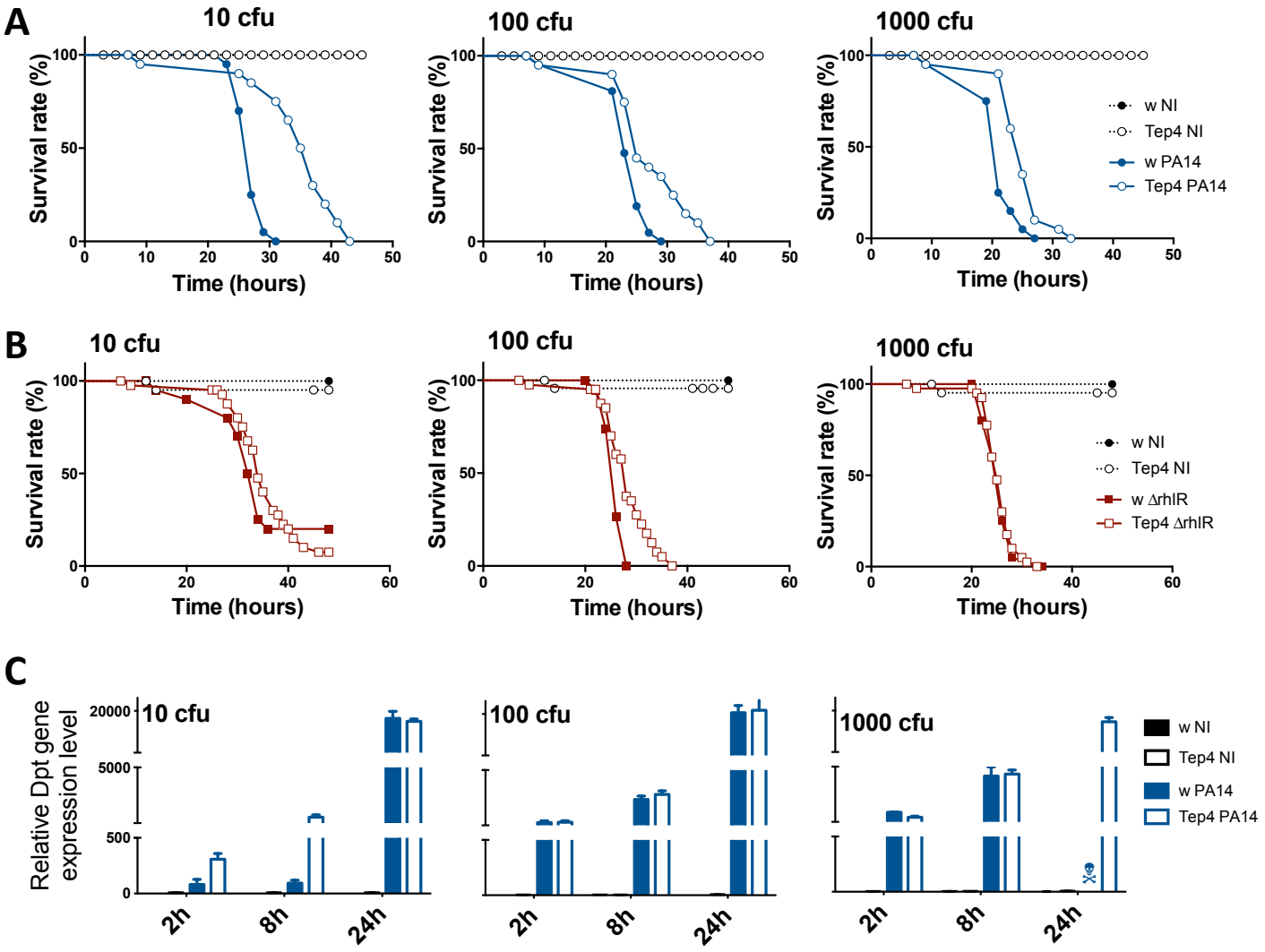

A
Fraction Worm Survival
1.0
0.8
0.6
0.4
0.2
0.0
20
40
60
80
100
120
Age at Death in Hours
WT PA14
ΔrhlI
ΔrhlR
B
C
1
PA14
2
ΔrhlI
3
ΔrhlR
D
E
Colonization of Bacteria in C.elegans Intestine
Relative Fluorescence Units
2.5x10^6
2x10^6
1.5x10^6
1x10^6
5x10^5
0
NS
*
**
n=18
n=15
n=18
n=16
n=15
n=13
PA14
ΔrhlI
ΔrhlR

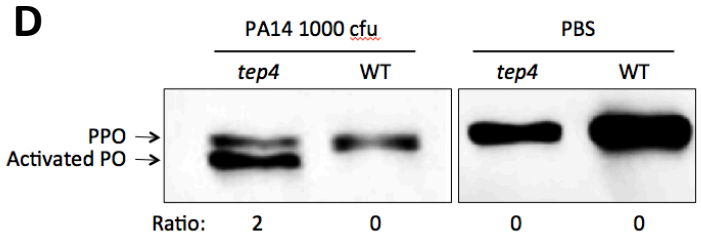

A
Gut
5 days
4d PA14-dsRed
1d PA14-GFP
6 days
4d PA14-dsRed
2d PA14-GFP
CFU/fly
10 000
1 000
100
10
1
PA14-GFP
PA14-dsRed
PA14-GFP
PA14-dsRed
B
Hemolymph
5 days
4d PA14-dsRed
1d PA14-GFP
6 days
4d PA14-dsRed
2d PA14-GFP
CFU/fly
100 000
10 000
1 000
100
10
1
PA14-GFP
PA14-dsRed
PA14-GFP
PA14-dsRed
C
w-PBS
w-LxB
w
LT50 (days)
-1 d
+4 h
+1 d
+4 d
+6 d
D
LT50 (days)
-1 d
+4 h
+1 d
+4 d
+6 d
E
LT50 (days)
-1 d
+4 h
+1 d
+4 d
F
PA14 WT
ΔrhlR
ΔrhlI
LT50 (w-w-LxB)
-1 d
+4 h
+1 d
+4 d
G
hmlGFP
4h
PA14
ΔrhlR
ΔrhlI
3d
PA14
ΔrhlR
ΔrhlI
H
hml-GFP+ cells / gut
NI
PA14
ΔrhlR
ΔrhlI
0
4h
1d
3d


A
Survival rate (%)
Time (days)
wA5001
Tep3
Tep4
Tep2,3
Tep2,3,4
B
Survival rate (%)
Time (days)
wA5001
Tep3
Tep4
Tep2,3
Tep2,3,4
C
Survival rate (%)
Time (days)
PA14 WT
ΔrhlR
ΔrhlI
NI
D
LT50 (days)
PA14 WT
ΔrhlR
ΔrhlI
**
E
LT50 w - LT50 Tep4 (days)
PA14 WT
ΔrhlI
ΔrhlR
***
***


A
w
Tep4
Phagocytosis index
500
400
300
200
100
0
*
*
PA14 WT
ΔrhlR
B
w
Tep4
Phagocytosis index
400
300
200
100
0
*
*
*
PA14 WT
ΔrhlR
ΔrhlI
PA14 WT
ΔrhlR
ΔrhlI


A
WT larvae
Tep4 larvae
Tep4 opsonin
Bacterial solution
Injection
WT larvae
Tep4 larvae
B
Phagocytosis index
1200
900
600
300
0
w
Tep4
PA14 WT
ΔrhlR
ΔrhlI
C
Phagocytosis index
20
15
10
5
0
PA14 WT
D
Phagocytosis index
1200
900
600
300
0
w
Tep4
PA14 WT
ΔrhlR
ΔrhlI
E
Phagocytosis index
20
15
10
5
0
PA14 WT


A
10 cfu
100 cfu
1000 cfu
Survival rate (%)
Time (hours)
w NI
Tep4 NI
w PA14
Tep4 PA14
B
10 cfu
100 cfu
1000 cfu
Survival rate (%)
Time (hours)
w NI
Tep4 NI
w ΔrhlR
Tep4 ΔrhlR
C
Relative Dpt gene expression level
10 cfu
100 cfu
1000 cfu
2h
8h
24h
w NI
Tep4 NI
w PA14
Tep4 PA14


D
PA14 1000 cfu
PBS
tep4
WT
tep4
WT
PPO
Activated PO
Ratio:
2
0
0
0