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* strain PA14, some bacteria cross the intestinal barrier and start proliferating 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 these bacteria to elude the cellular immune response. RhlI synthesizes the auto-inducer signal that activates RhlR. Here, we compare the null mutant phenotypes of *rhlR* and *rhlI* in a variety of infection assays in *Drosophila* and in the nematode *Caenorhabditis elegans*. Surprisingly, in *Drosophila,* 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 *C. 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, which also depends on *rhlR*. 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, Ferrandon, 2013, Igboin, Griffen et al., 2012, Limmer, Quintin et al., 2011b). Genetic analysis has allowed the detailed dissection of the *Drosophila*

52 systemic immune response to microbial infections (Lemaitre & Hoffmann, 2007). In addition
53 to melanization which is mediated by the protease-mediated cleavage of prophenol oxidase
54 into active phenol oxidase (PO), two major NF-kappaB pathways, Toll and Immune
55 deficiency (IMD), regulate the induction of the expression of genes that encode potent
56 antimicrobial peptides, which are active against most bacteria and fungi (Binggeli, Neyen et
57 al., 2014, Dudzic, Kondo et al., 2015, Ferrandon, Imler et al., 2007, Ganesan, Aggarwal et al.,
58 2010, Lemaitre & Hoffmann, 2007). The *Drosophila* systemic immune response is so
59 effective, especially in the case of Gram-negative bacterial infections, that a second arm of
60 host defense, the cellular immune response, has remained comparatively less well studied (El
61 Chamy, Matt et al., 2017, Pean & Dionne, 2014). Indeed, blocking cellular immunity through
62 saturation of the phagocytic apparatus with inert particles does not yield a strong
63 susceptibility phenotype to flies infected by *Escherichia coli*, unless the systemic immune
64 response is at least also partially impaired (Elrod-Erickson, Mishra et al., 2000). Nevertheless,
65 we have found that when two opportunistic pathogens *Serratia marcescens* and *Pseudomonas*
66 *aeruginosa* are fed to *Drosophila*, the cellular immune response plays a key role in
67 controlling the bacteria that escape from the digestive tract (Limmer, Haller et al., 2011a,
68 Nehme, Liegeois et al., 2007). In both cases, the putative phagocytic receptor Eater plays a
69 crucial role and prevents the development of a rapid bacteremia (Kocks, Cho et al., 2005,
70 Limmer et al., 2011a).

71 The thioester-containing protein Tep1 opsonizes bacteria in the mosquito species *Anopheles*
72 *gambiae* (Levashina, Moita et al., 2001). It is unknown whether opsonization also plays a role
73 *in vivo* in *Drosophila*, even though its genome encodes five functional *Tep* loci and a
74 pseudogene (*Tep5*) (Bou Aoun, Hetru et al., 2011). The thioester motif is not present in Tep6
75 and is therefore thought to be nonfunctional. Indeed, Tep6 is required for the establishment of
76 septate junctions in specific parts of the gut, which explains the lethal phenotype of *Tep6* null

77 mutants (Batz, Forster et al., 2014, Hall, Bone et al., 2014). Tep6 has also been shown to
78 induce autophagy in macrophages via a non cell-autonomous process that involves epithelial
79 cells in which it is expressed (Lin, Rodrigues et al., 2017). A previous study failed to find a
80 role for the remaining *Tep* genes (*Tep1* to *Tep4*) in host defense in several models of bacterial
81 or fungal systemic infections (Bou Aoun et al., 2011), although a study reported that *Tep3*
82 mutant flies are highly susceptible to the nematode parasite *Heterorhabditis bacteriophora*
83 (Arefin, Kucerova et al., 2014). Interestingly, a study led in *Drosophila* cultured S2 cells
84 showed that *Tep2* is required for the phagocytosis of the Gram-negative species *E. coli*, *Tep3*
85 is required for the uptake of the Gram-positive *Staphylococcus aureus*, and unexpectedly that
86 *Tep6* is required to phagocytose the dimorphic yeast *Candida albicans* (Stroschein-Stevenson,
87 Foley et al., 2006).

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

98 RhlR is the major regulator of one of the three known quorum-sensing systems in *P.*
99 *aeruginosa*. Quorum-sensing systems play a major role in coordinating the expression of
100 virulence genes in several infection models (Coggan & Wolfgang, 2012, Jimenez, Koch et al.,
101 2012, Schuster, Sexton et al., 2013, Williams & Camara, 2009). However, we have failed to

102 uncover a strong role for the other two *P. aeruginosa* quorum sensing systems regulated by
103 LasR and MvfR in the *Drosophila* intestinal infection model (Limmer et al., 2011a). This
104 observation was somewhat unexpected since the LasR-mediated quorum-sensing system
105 appears to function upstream of the RhlR-mediated system. RhlR is activated by binding to an
106 auto-inducer molecule, butanoyl-homoserine lactone (C4-HSL), which is synthesized by the
107 RhlI enzyme. The transcription of the *rhlI* and *rhlR* genes is in turn activated by the Las
108 transcriptional regulator LasR (Coggan & Wolfgang, 2012, Jimenez et al., 2012, Schuster et
109 al., 2013, Williams & Camara, 2009). Activation of RhlR takes place when a threshold
110 concentration of C4-HSL is reached, which correlates with a threshold bacterial
111 concentrations reached during exponential growth in *in vivo* studies.

112 Here, we report that the virulence phenotype exhibited by ingested PA14 *rhlI* mutants is
113 strikingly distinct from that exhibited by *rhlR* mutants. *rhlR*mutants exhibit severely impaired
114 virulence, whereas *rhlI* mutants at most modestly impaired virulence, suggesting that RhlR
115 can function independently of activation by C4-HSL. We further establish that RhlR, but not
116 RhlI, is required to elude opsonization by a *Tep4*-dependent process. Finally, we establish that
117 in contrast to its protective role during PA14 intestinal infections, Tep4 plays the opposite
118 role in a systemic infection model, possibly by preventing the activation of phenol oxidase.

119 **RESULTS**

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

121 RhlR is activated by C4-HSL synthesized by the RhlI enzyme, as shown in numerous *in vitro*
122 studies (Gambello, Kaye et al., 1993, Latifi, Winson et al., 1995, Pesci, Pearson et al., 1997,
123 Seed, Passador et al., 1995). Thus, we expected that *rhlI* and *rhlR* mutants would display the
124 same phenotype. Unexpectedly, however, although a *ΔrhlI* deletion mutant strain was indeed
125 less virulent than wild-type PA14 when ingested by flies, it was significantly more virulent

126 than *ΔrhlR* (Fig. 1A, B). Moreover, whereas the *ΔrhlR* mutant was cleared from the
127 hemolymph, the *ΔrhlI* mutant proliferated in this compartment, although it appeared to grow
128 less rapidly than wild-type PA14 (Fig. 1C, D). Consistent with this latter result, *ΔrhlI* but not
129 *ΔrhlR* triggered a systemic immune response, as monitored by measuring the expression of
130 the *Diptericin* gene (Fig. S1E-F). Similar results were obtained with independent *ΔrhlR* and
131 *ΔrhlI* in frame deletion mutants constructed by another laboratory (Fig. S1) (Hoyland-
132 Kroghsbo, Paczkowski et al., 2017), thereby confirming the correlation between the *ΔrhlR*
133 and *ΔrhlI* null genotypes and the differing *ΔrhlR* and *ΔrhlI* phenotypes.

134 We next tested the survival rates of flies in which the cellular response had been ablated by
135 injecting latex beads (LXB) after feeding on wild-type or *ΔrhlR* or *ΔrhlI* mutant bacteria.
136 Both *ΔrhlR* and *ΔrhlI* killed latex bead-injected flies much faster than PBS-injected control
137 flies, at approximately the same rate, but more slowly than wild-type PA14, with the
138 difference between *ΔrhlI* and wild-type PA14 at the borderline of statistical significance
139 (p=0.07) (Fig. 1E, F).

140 It is important to determine whether the apparently enhanced virulence of *ΔrhllR* and *ΔrhlI*
141 mutants observed in phagocytosis-impaired flies (compared to flies not injected with latex
142 beads) is simply a reflection of the increased virulence of wild-type PA14 in these immuno-
143 deficient flies, or whether the enhanced virulence of the *ΔrhlR* and *ΔrhlI* mutants is indicative
144 of the fact that the RhlR-mediated regulatory systems plays an important role in counteracting
145 the cellular immune response. To this end, it is useful to measure the difference in LT_{50} values
146 of control *vs*. latex bead-injected flies (LT_{50}[wt-wt^{LXB}]) for each mutant and to compare it to
147 that measured for wild-type PA14. *ΔrhlR* did recover virulence with a LT_{50}[wt- wt^{LXB}] of 4.7
148 days, as compared to 2.4 days for wild-type PA14, which corresponds to the level of
149 recovered virulence reported earlier (Fig. 1G) (Limmer et al., 2011a). With a value of 3.5

150 days, *ΔrhlI* displayed an intermediate LT_{50}[wt- wt^{LXB}], although the significance of the
151 difference with wild-type PA14 or *ΔrhlR* could not be assessed as the *ΔrhlI* values were too
152 spread out (Fig. 1G). Indeed, *ΔrhlI* mutants consistently tended to display a more variable
153 survival phenotype than the *ΔrhlR* mutant (Fig. 1B).

154 Finally, both the *ΔrhlR* and *ΔrhlI* mutant strains yielded similarly shaped survival curves
155 when used to infect flies with an impaired cellular defense. These survival curves were less
156 steep than those obtained with wild-type PA14, as measured by their Hill coefficients (Fig.
157 1H), suggesting that quorum-sensing is involved in determining the shape of the survival
158 curve. This reflects a collective property of flies placed in the same vial, which succumb less
159 synchronously during infections when the *rhl* quorum sensing system is missing.

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

161 The nematode *C. elegans* is a well-established model host to study *P. aeruginosa*
162 pathogenesis (Irazoqui, Urbach et al., 2010, Pukkila-Worley & Ausubel, 2012, Tan, Mahajan-
163 Miklos et al., 1999a). The *C. elegans* intestinal infection model shares some key features with
164 the *Drosophila* model, at least during the initial stages of the infection. For instance, in both
165 models, ingested bacteria are exposed in the gut lumen to antimicrobial peptides and to
166 reactive oxygen species generated by the Dual oxidase enzyme {Ha, 2005 #1857}{Chavez,
167 2009 #3616}. However, in contrast to ingested *P. aeruginosa* in *Drosophila*, PA14 are not
168 known to escape from the gut compartment during the nematode infection. We therefore
169 tested whether the differences that we observed in the virulence of *ΔrhlR* and *ΔrhlI* mutants in
170 the *Drosophila* intestinal infection assay were reflected in a well-established *C. elegans – P.*
171 *aeruginosa* nematode "slow killing" survival assay (McEwan, Feinbaum et al., 2016, Tan et
172 al., 1999a). Indeed, two independently constructed in frame *rhlR* deletion mutants in the

173 PA14 background were dramatically less virulent than two independently constructed *rhlI*
174 deletion mutants in their ability to kill *C. elegans* (Fig. 2A)

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

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

193 In the *Drosophila* intestinal infection model, flies constantly feed on PA14 present on a filter.
194 A defining feature of this model is that even though bacteria are able to rapidly cross the
195 intestinal barrier to penetrate the hemocoel, as had been previously described for *Serratia*
196 *marcescens* (Nehme et al., 2007), the PA14 titer in the hemolymph remains low for the first

197 few days of the infection. After this initial incubation period, there is an exponential
198 proliferation of PA14 in the hemocoel, which coincides with the activation of the systemic
199 immune response. Using bacteria expressing different colored fluorescent proteins, we
200 previously showed that *S. marcescens* continuously crosses the intestinal barrier during the
201 infection (Nehme et al., 2007). Fig. 3A-B shows that when flies that have been feeding on
202 dsRed-labeled PA14 bacteria for four days were switched to a filter laced with GFP-labeled
203 PA14, the green bacteria progressively replaced the red bacteria both in the gut and hemocoel
204 compartments. We conclude that *P. aeruginosa*, like *S. marcescens*, continuously crosses the
205 intestinal barrier during the infection.

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

219 In contrast to wild-type PA14, *ΔrhlR* bacteria were kept in check by phagocytosis at least up
220 to day four and to some extent up to six days after infection (Fig. 3D, F). Phagocytosis was
221 efficient against *ΔrhlI* bacteria for approximately four days (Fig. 3E, F). These data suggest

222 that *ΔrhlR* bacteria are constantly kept in check by the cellular immune response when
223 penetrating the hemocoel, whereas wild-type PA14 ultimately escape this immune
224 surveillance. *ΔrhlI* bacteria display an intermediate phenotype, with a somewhat decreased
225 virulence in flies in which phagocytosis was blocked at day 4 (Fig. 3F).

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

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

235 Our previous work had shown that flies mutant for the putative phagocytic receptor gene
236 *Eater* are more susceptible to PA14 ingestion and display a phenotype similar to that obtained
237 by latex bead-mediated ablation of the phagocytic capacity of hemocytes (Limmer et al.,
238 2011a). Thioester-containing proteins have been reported to be required for phagocytosis in
239 mosquitoes and also in cultured *Drosophila* S2 cells (Levashina et al., 2001, Stroschein-
240 Stevenson et al., 2006). We therefore tested mutations affecting the *Tep2*, *Tep3*, and/or *Tep4*
241 genes (Bou Aoun et al., 2011). In the case of *Tep1*, since no mutants were available, we tested
242 an RNAi transgene expressed either in hemocytes or in the fat body. However, we did not
243 observe any change in the virulence of ingested PA14 (Fig. S4A, B). *Tep4* and triple *Tep2-*
244 *Tep3-Tep4* mutants displayed increased susceptibility to PA14 ingestion, in contrast to *Tep3*
245 and double *Tep2-Tep3* mutants that displayed respectively a somewhat decreased or wild-type

246 susceptibility (Fig. 4A, D-E). Of note, uninfected *Tep3* mutants fed on a sucrose solution
247 displayed an enhanced fitness when compared to wild-type or other *Tep* mutant lines (Fig.
248 S4C). We conclude from these data that Tep4, but not other thioester-containing proteins, is
249 required for host defense against ingested PA14.

250 Next, we found that *ΔrhlR* became as virulent as wild-type PA14 when ingested by *Tep4* or
251 *Tep2-Tep3-Tep4* mutants, which was not observed with the *Tep2* and *Tep2-Tep3* mutant
252 strains (Fig. 4B, D-E). Interestingly, the injection of latex beads in *Tep4* flies only modestly
253 increased the virulence of *rhlR* bacteria when compared to PBS-injected *Tep4* flies (Fig. S4 D,
254 E), suggesting that phagocytosis of PA14 is severely affected in the *Tep4* mutant. *ΔrhlI*
255 bacteria behaved like *ΔrhlR* bacteria when ingested by *Tep4* (Fig. 4C, E), similarly to flies
256 injected with latex beads (Fig. 1E), although *ΔrhlR* recovered virulence to a much larger
257 extent (3.1 days) than *ΔrhlI* (0.9 days) when ingested by *Tep4* flies. Hence, the behavior of
258 *ΔrhlR* mutant PA14 is similar in *eater* and *Tep4* mutant flies, thereby raising the possibility
259 that both fly genes are involved in the same process, in keeping with an unchanged phenotype
260 of *Tep4* when phagocytosis was blocked by the injection of latex beads (Fig. S4DC).

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

262 To quantitatively monitor the uptake of PA14, we used assays that relied on larval hemocytes.
263 First, we injected heat-killed, pHrodo®-labeled bacteria in wild-type or *Tep4* third-instar
264 larvae. The dye becomes fluorescent when placed in an acidic environment such as that
265 encountered in phagosomes. After 45 minutes, the larvae were bled and a phagocytic index
266 was established. Wild-type hemocytes ingested significantly more PA14 or *ΔrhlR* bacteria
267 than *Tep4* hemocytes (Fig. 5A). There were however no significant differences between heat-
268 killed wild-type PA14 and *rhlR* bacteria uptake by wild-type hemocytes on the one hand, or
269 *Tep4* hemocytes on the other (Fig. 5A). This was not necessarily unexpected as heat-killing

270 likely inactivates *rhlR*-dependent virulence factors and might also alter the surface of bacteria.
271 We therefore modified the assay with live bacteria and used an antibody we had raised against
272 PA14 to differentially immuno-stain the bacteria, prior to and after permeabilization of the
273 fixed cells. As before, *Tep4* hemocytes exhibited a decreased uptake of bacteria compared to
274 wild-type hemocytes. However, in the case of live bacteria, *ΔrhlR* bacteria were significantly
275 better phagocytosed than wild-type PA14 bacteria when injected into wild-type or *Tep4* larvae.
276 *ΔrhlI* exhibited an intermediate phenotype in this assay and was not significantly different
277 from either wild-type or *ΔrhlR* bacteria (Fig. 5B). We conclude that this assay is not sensitive
278 enough to discriminate between these two bacterial mutant strains. We obtained similar
279 results using the *ΔrhlR* and *ΔrhlI* strains generated by another laboratory (Fig. S1G).

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

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

294 6E), which was nevertheless lower than that of bacteria that had been pre-incubated with
295 wild-type hemolymph (Fig. 6C). When these experiments were performed using *ΔrhlR*
296 bacteria that were injected in *Tep4* larvae, it made a major difference whether these mutant
297 bacteria had first been pre-exposed to wild-type or *Tep4* hemolymph. Tep4-dependent
298 opsonization led to a massive uptake of bacteria (median phagocytic value of 162), whereas
299 nonopsonized bacteria (pre-incubated with *Tep4* mutant hemolymph) were hardly ingested
300 (median phagocytic value of 12; Fig. 6D-E). As expected, nonopsonized bacteria that were
301 then injected in wild-type recipients were much better phagocytosed (median phagocytic
302 value of 214), presumably because Tep4 was circulating in the wild-type hemolymph (Fig.
303 6B). They were nevertheless ingested less efficiently than opsonized bacteria (median
304 phagocytic value of 376; Fig. 6B). Finally, *ΔrhlI* bacteria were also opsonized by Tep4, but
305 significantly less than *ΔrhlR* bacteria (Fig. 6B, D). Again, they displayed a phenotype that
306 was intermediate to that of wild-type PA14 on the one hand, and *ΔrhlR* on the other. We
307 conclude that PA14 and, to a lesser extent, *ΔrhlI* bacteria are less efficiently opsonized and
308 subsequently phagocytosed than *ΔrhlR* bacteria, which are therefore unable to elude the
309 cellular immune response.

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

311 A recent study has reported that *Tep4* mutants are more resistant to a systemic infection with
312 the entomopathogenic bacterium *Photorhabdus luminescens* in a septic injury model (Shokal
313 & Eleftherianos, 2017). By injecting several doses of PA14, from 10 to 1000 CFUs, directly
314 into the thorax of flies, we also consistently found that *Tep4* mutants survived better than
315 wild-type flies in this systemic infection model (Fig. 7A). This difference in survival between
316 *Tep4* and wild-type flies was largely attenuated when *ΔrhlR* bacteria were injected, thereby
317 establishing again a relationship of altered virulence of these bacteria in a *Drosophila Tep4*

318 mutant background (Fig. 7B). Using the steady-state expression of the antibacterial peptide
319 gene *Diptericin* as a read-out of the activation of the IMD pathway that regulates the systemic
320 immune response against Gram-negative bacteria, we found no significant difference of
321 expression between wild-type and *Tep4* (Fig. 7C). As a higher level of induction of the IMD
322 pathway is unlikely to account for the increased resistance of *Tep4* mutants against PA14
323 infection, we tested whether the phenol oxidase cascade was more efficiently activated in this
324 mutant background, as previously reported (Shokal & Eleftherianos, 2017). Indeed, we found
325 that pro-phenol oxidase was cleaved to some extent in *Tep4* but not in wild-type flies. These
326 data further suggest that wild-type PA14 bacteria elude detection by the factors that trigger
327 the prophenol oxidase cascade, and that Tep4 plays a role in this process of evasion from the
328 melanization response.

329 **DISCUSSION**

330 In this study, we analyzed the interactions of *P. aeruginosa* with *Drosophila* from the dual
331 perspective of both pathogen and host. Our data lead us to propose a model in which RhlR
332 plays a pivotal role in virulence by diminishing the ability of the cellular immune arm of the
333 host defense response to detect *P. aeruginosa* once the bacteria have reached the internal
334 body cavity of the insect after crossing the intestinal barrier. Surprisingly, RhlR function in
335 eluding opsonization by Tep4 is at least partially independent of the C4-HSL producing
336 enzyme RhlI. These results as well as those of another study (Mukherjee, Moustafa et al.,
337 2017) show that RhlR can function independently of C4-HSL, but they do not formally
338 establish that RhlR is functioning independently from a quorum-sensing system in this
339 function. Furthermore, we establish a dual function for the putative opsonin Tep4, which
340 plays opposite roles in host defense depending on the infection route.

341

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

343 *P. aeruginosa* is a pathogen that uses complex signaling mechanisms to adapt to its
344 environment, and its three quorum-sensing regulators (LasR, RhlR and MvfR) appear to be
345 involved in the regulation of a variety of virulence-related functions (Coggan & Wolfgang,
346 2012, Jimenez et al., 2012, Schuster et al., 2013). *In vitro* studies, sometimes complemented
347 by *in vivo* experiments, have revealed that these quorum-sensing systems are intricately
348 intertwined. It was thus unexpected that only the *P. aeruginosa* RhlR regulator appears to
349 play a critical role for virulence in a *Drosophila* intestinal infection model and not the two
350 other quorum sensor regulators (Limmer et al., 2011a). Here, we report that *ΔrhlR* null
351 mutants consistently display virulence levels that are much weaker than those observed with
352 *ΔrhlI* mutants (Fig. 1B), do not proliferate in the hemolymph in contrast to *ΔrhlI* mutants, and
353 are phagocytosed and opsonized in a Tep4-dependent process more efficiently than *ΔrhlI* and
354 wild-type bacteria. These observations suggest that RhlR functions at least partially
355 independently from RhlI and presumably independently from C4-HSL activation. In contrast,
356 *ΔrhlI* mutants exhibit an impaired virulence in both wild-type and *Tep4* immuno-deficient
357 flies, similarly to *ΔrhlR* mutants. Both *ΔrhlI* and *ΔrhlR* mutants display survival curves with
358 shallow slopes. Thus, the partially overlapping phenotypes of *ΔrhlI* and *ΔrhlR* in flies with
359 impaired cellular immunity suggests that RhlR partially functions together with RhlI as a
360 classical quorum-sensing regulator in a process that remains to be delineated. Interestingly, it
361 appears that RhlR controls gene expression for biofilm formation both in a C4-HSL-
362 dependent and C4-HSL independent manner (Mukherjee et al., 2017). However, this putative
363 conventional function of RhlR plays a minor role in virulence of PA14 in *Drosophila*, as
364 shown by the weak virulence-related phenotypes of *ΔrhlI* mutants documented in our study.

365 One explanation for the RhlI-independent activity of RhlR is that it gets activated in a C4-
366 HSL-independent manner by an as yet unidentified quorum-sensing compound. Of note, RhlR
367 does not appear to be activated by 3-oxo-C12-HSL (Mukherjee et al., 2017), the LasR ligand.
368 In any case, *lasR* and *lasI* mutant bacteria display only a modestly decreased virulence
369 phenotype in the *Drosophila* infection model (Fig. S5). The diketopiperazines (DKPs)
370 represent a candidate family of RhlR-activating compounds (Holden, Ram Chhabra et al.,
371 1999); however, at least one study failed to detect any interaction of these compounds with
372 LuxR proteins (Campbell, Lin et al., 2009). The resolution of this issue will require testing
373 mutants that affect the synthesis of DKPs.

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

384 Finally, our studies on the inactivation of the cellular immune response at different time
385 points of the infection further support a quorum sensing-independent role of RhlR. Our study
386 revealed that phagocytosis is required only when very few bacteria are present in the
387 hemolymph, that is, during the first days of the infection. A caveat here is that we cannot
388 exclude the possibility that C4-HSL or other cryptic autoinducers might be produced by the
389 bacteria present in large numbers in the gut compartment. However, if autoinducers, including

390 C4-HSL, were produced in the intestinal lumen and were able to cross the digestive barrier, it
391 is difficult to understand why they would not immediately activate RhlR resulting in full-
392 blown bacteremia without the observed lag before the exponential proliferation phase in the
393 hemocoel. This hypothesis also does not account for why the *rhlI* reduced virulence
394 phenotype is much weaker than that of *rhlR*, unless this reflects the differing opsonization
395 properties of these mutants.

396 ***The function of RhlR in bypassing host defenses in* C. elegans?**

397 Except for fungal invasion of the epidermis by nematophagous fungi, how *C. elegans* senses
398 infections remains poorly understood (Zugasti, Bose et al., 2014). The finding that *ΔrhlR*
399 mutants are much less virulent than Δ*rhlI* mutants in a *C. elegans* infection model, and that
400 *ΔrhlR* mutants fail to colonize the intestinal tract of worms might be due either to an impaired
401 escape from detection by the immune system or to a defective resistance to its action. It is not
402 clear, however, that RhlR-mediated regulation of the production of toxic phenazines, as
403 shown in the case of PA14-mediated "fast killing" of *C. elegans* (Mukherjee et al., 2017), is
404 the reason the *ΔrhlR* mutant is so dramatically impaired in the *C. elegans* "slow killing" assay
405 used in our study in Fig. 2A. The major difference in the fast and slow *C. elegans* killing
406 assay is the composition of the agar medium in which the *P. aeruginosa* is grown and on
407 which the killing assays are performed (Mahajan-Miklos, Tan et al., 1999, Tan et al., 1999a,
408 Tan, Rahme et al., 1999b). Fast killing is mediated by phenazines (Cezairliyan, Vinayavekhin
409 et al., 2013, Mahajan-Miklos et al., 1999) whereas slow killing is multi-factorial (Feinbaum,
410 Urbach et al., 2012), and PA14 mutants deficient in the production of phenazines do not
411 exhibit a significant killing defect in the slow killing assay (Tan et al., 1999b). In contrast to
412 *Drosophila*, no cellular host defense has been detected in *C. elegans* and is unlikely to be
413 involved in the immune response to intestinal infection. The use of *ΔrhlR* mutants will open

414 the way to the identification of the relevant host defense systems that are circumvented by
415 wild-type PA14 bacteria. In any case, the lack of a significant phenotype of the *ΔrhlI* mutants
416 in the *C. elegans* killing and intestinal proliferation assays is striking. This can be exploited in
417 future studies to help elucidate the underlying *rhlI*-independent mechanisms involved in
418 RhlR-mediated regulation of virulence.

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

420 Our phagocytosis and opsonization data are consistent with a model in which RhlR controls
421 the expression of gene products that mask the site being recognized by Tep4 or a Tep4-
422 associated protein, presumably on the cell wall. Alternatively, RhlR may actively inhibit the
423 uptake of opsonized bacteria. The masking or inhibition of ingestion processes may be
424 sensitive to heat, as wild-type heat-killed bacteria appeared to be more efficiently taken up by
425 hemocytes than live ones (compare median values for PA14WT in Fig. 5A to those in 5B).
426 Alternatively, the processes may be unstable and require permanent maintenance that can no
427 longer be achieved when the bacterial cells are killed.

428 Insect thioester-containing proteins belong to the complement family, and have been shown to
429 be involved in the opsonization of bacteria in mosquitoes. In *Drosophila*, Tep2 has been
430 reported to be required for the uptake of *Escherichia coli*, a Gram-negative bacterium, by
431 cultured S2 cells (Stroschein-Stevenson et al., 2006), a finding confirmed *in vivo* (Shokal,
432 Kopydlowski et al., 2017). In contrast, we find no involvement of Tep2 in our *in vivo*
433 intestinal infection model with *P. aeruginosa* but do detect a requirement for Tep4 in
434 phagocytosis and opsonization assays. Given that the structure of mosquito thioester-
435 containing protein 1 is similar to that of complement family C3, a well-described opsonin, our
436 data are compatible with a model of direct opsonization of bacteria by Tep4.

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

439 The finding that Tep4 plays a protective function in the intestinal infection model whereas it
440 is detrimental in the case of a direct systemic infection is paradoxical. This may actually
441 represent two faces of the same phenomenon. PA14 may have developed a stealth strategy to
442 avoid detection by the immune system of living organisms and thus may actively hide any
443 features that might reveal its presence. We propose here that RhlR plays a critical role in a
444 program that renders PA14 furtive, in keeping with our finding that bacteria likely need to be
445 alive to escape phagocytosis efficiently (Fig. 5). As a result of RhlR action, only a few sites
446 would be available on the surface of the wild-type bacteria for Tep4 direct or indirect binding.
447 There, Tep4 would mediate opsonization and then subsequent phagocytosis of the bacteria.
448 Our data in the systemic infection model are compatible with the possibility that Tep4
449 competes for these sites with Pattern Recognition Receptors (PRRs) that activate the
450 prophenol oxidase cascade since PO activation occurs only in the *Tep4* mutant background. It
451 is likely that small peptidoglycan (PGN) fragments released by proliferating bacteria
452 represent a major trigger of the IMD pathway in addition to large PGN fragments directly
453 sensed by PGRP-LC, thereby accounting for the apparent normal expression of *Diptericin*
454 when flies are challenged with injected PA14. In contrast, we have previously established that
455 some fungi and Gram-positive bacteria trigger the phenol oxidase cascade through defined
456 PRRs (Matskevich, Quintin et al., 2010). The situation is less clear as regards Gram-negative
457 bacteria. While the original characterization of PGRP-LE suggested that it triggers the phenol
458 oxidase activation cascade (Takehana, Katsuyama et al., 2002), and acts non cell-
459 autonomously (Takehana, Yano et al., 2004), subsequent studies have documented a role for
460 PGRP-LE as an intracellular sensor for PGN fragments (Bosco-Drayon, Poidevin et al., 2012,

461 Ferrandon et al., 2007, Yano, Mita et al., 2008). Thus, further work will be required to
462 identify whether Tep4 actually competes with PRRs in the detection of pathogens.

463 Our results further suggest that the cellular immune response is a relevant defense when a few
464 bacteria enter the hemocoel after escaping from the digestive tract in the intestinal infection
465 model, but not in the septic injury model. Conversely, melanization mediated by activated
466 phenol oxidase appears to be somewhat effective after injection but not ingestion of PA14.

467 A major challenge will be to establish how RhlR influences the surface properties of PA14 or
468 alternatively inhibits the uptake of opsonized bacteria.

469 Finally, *ΔrhlR* mutants exhibit reduced dissemination capacities in a rodent lung infection
470 model when compared to *ΔrhlI* or wild-type PA14(Mukherjee et al., 2017). By analogy to our
471 findings in the *Drosophila* intestinal infection model, it will be interesting to determine
472 whether the complement system restricts the systemic escape of *ΔrhlR* mutants from the
473 mouse lung into the periphery.

474 **MATERIALS AND METHODS**

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

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

477 The "slow-killing" of *C. elegans* by *P. aeruginosa* was monitored using automated image
478 analysis as previously described (McEwan et al., 2016, Stroustrup, Ulmschneider et al.,
479 2013). To monitor the accumulation of *P. aeruginosa* in the *C. elegans* intestine, wild-type
480 (N2) animals were used for all experiments. Worms were reared on non-pathogenic *E. coli*
481 *OP50* on nematode growth media at 25°C. Synchronized L4 worms (fourth larval stage) were
482 transferred to slow kill (SK) nematode growth media agar plates containing a lawn of *P.*
483 *aeruginosa* PA14::GFP strains. Post infection at 24 and 48 hours approximately 20 worms

484 were picked onto a 2% agar pad that contain the paralyzing agent levamisole (1mM). The
485 worms were imaged in the GFP channel using a Zeiss Apotome microscope with the same
486 exposure time for all the worms on wild type PA14 and the ***Δ****rhlR* and ***Δ****rhlI* mutants. Post
487 acquisition the images were processed using ImageJ software and the area and fluorescence
488 intensity was measured. The relative fluorescence intensity is plotted and a non-parametric
489 Mann-Whitney test was used to determine statistical significance.

490 **Opsonization assay of live bacteria**

491 Overnight cultures of PA14, *ΔrhlR*, and *ΔRhlI* mutants were concentrated to OD10 in PBS.
492 Twenty third instar larvae were bled in 150 µL of bacterial resuspended in PBS at aOD of 10
493 and incubated at room temperature for 30 to 45 min. Samples were centrifuged at 500 rcf for
494 15 min and the pellet (containing larval debris) was removed. A second centrifugation was
495 performed at 3500 rcf for 15 min to retrieve bacteria in the pellet, that was re-suspended in
496 10 µL PBS. A5001 or *tep4* third instar larvae were injected with 32.2 nL of the live bacteria
497 solutions, using a Nanoject apparatus (Drummont). After 60-90 min of incubation, one larva
498 was bled in each well of an 8-well pattern microscopy slide that contained PBS. The cells
499 were left to settle to the bottom for 30 min and were then fixed in 4 % paraformaldehyde for
500 15 min, in a humid chamber. The samples were washed twice in PBS and they were stained
501 with a 1/500 diluted rabbit antiserum against PA14 in a PBS solution with 2 % BSA for 2
502 hours at room temperature. The cells were incubated with a FITC-labeled goat anti-rabbit
503 secondary antibody (Invitrogen) in a PBS solution with 2 % BSA for 2 hours at room
504 temperature. After a 20 min permeabilization step in a PBS solution with 0.1 % Triton X-100
505 and 2 % BSA, a second round of staining with a 1/500 diluted rabbit antiserum against PA14
506 in a PBS solution with 0.1 % Triton X-100 and 2 % BSA was performed for 2 hours at room
507 temperature. The samples were then incubated with a Cy3-labeled goat anti-rabbit secondary
508 antibody (Invitrogen) in a PBS solution with 0.1 % Triton X-100 and 2 % BSA for 2 hours at

509 room temperature. The slides were mounted in Vectashield with DAPI (Vector Laboratories)
510 and analyzed using a Zeiss Axioskope 2 fluorescent microscope. 40 to 50 cells per larva were
511 analyzed: the number of red fluorescent bacteria that were not green fluorescent was counted
512 for each DAPI-positive hemocyte, and the phagocytosis index was calculated (% of
513 phagocytes containing at least 1 only-green bacterium)×(mean number of only-green bacteria
514 per positive cell). We used the nonparametric Mann-Whitney test for statistical analysis.
515

516 **Phenol oxidase cleavage assay**

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

522 **Statistical analysis**

523 All statistical analysis were performed on Graphpad Prism version 5 (Graphpad software Inc.,
524 San Diego, CA). Details are indicated in the legend of each figure.
525

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535

536 **AUTHOR CONTRIBUTIONS**

537 SH, AF, SL, and DF conceived the *Drosophila* experiments and analyzed the data. SH
538 performed the majority of these experiments, with later work performed by AF with an input
539 from SL; SS performed the precursor experiments that led to this work. Work on the septic
540 injury model was performed by JC, with some help from ZL. *ΔlasR*, ΔlasI, ΔrhlR and *ΔrhlI*
541 mutants were constructed by ED and SY, except when indicated otherwise. AH and FMA
542 conceived the *C. elegans* experiments and analyzed the data, which were obtained by AH. SH,
543 DF, and FMA wrote the manuscript, with inputs from other co-authors.

544 **CONFLICT OF INTEREST**

545 The authors report no conflict of interests.

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710 Pseudomonas aeruginosa: a tale of regulatory networks and multifunctional signal molecules.
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713 Fukase K, Silverman N, Yoshimori T, Kurata S (2008) Autophagic control of listeria through
714 intracellular innate immune recognition in drosophila. Nat Immunol 9: 908-16
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717 immune response of Caenorhabditis elegans. Nat Immunol 15: 833-8

718

719 **FIGURE LEGENDS**

720

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

723 Survival experiment of wild-type flies (*w*) fed on wild-type (WT) PA14 bacteria, or Δ*rhlR,* or
724 Δ*rhlI* mutants.

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

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

736 C-D. Bacterial titer of the hemolymph collected from flies that had ingested wild-type or
737 mutant PA14 as indicated, three (C) and five (D) days after the ingestion of PA14 WT or
738 mutants as indicated. In this series of experiments, flies infected with PA14 WT had started to
739 succumb by day five and were therefore not analyzed. Statistics were performed using the
740 Mann Whitney test; bars indicate medians. p values for C are respectively from left to right:
741 0.003, 0.002, and 0.005. P<0.0001 for D.

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

746 F. Pooled LT_{50} data of latex bead-injected flies (*w*-LXB) survival experiments. *w*-LxB flies
747 died significantly slower after Δ*rhlR* infection than with PA14 WT (**p=0.0065). A slight
748 decrease of virulence, but at the border of significance, was observed between PA14 WT and
749 Δ*rhlI* (p=0.0726). No difference in virulence was detected between Δ*rhlR* and Δ*rhlI*
750 (p=0.3056). Data represent the LT_{50}s from five experiments (biological duplicates are also
751 shown as there was as much variability between experiments as within experiments), bars
752 indicate medians. Statistics were performed using a non-parametric test (Mann Whitney).

753 G. Differences of LT_{50}s between WT flies pre-injected with PBS (WT) and flies pre-injected
754 with latex-beads (WT^{LXB}) after intestinal infection with PA14 WT or Δ*rhlR* mutant or Δ*rhlI*
755 mutants, bars indicate medians. There is only a significant difference between PA14 WT and
756 the Δ*rhlR* mutant (*p=0.0244) but not the Δ*rhlI* mutant. Data represent the LT50s from five
757 experiments (biological duplicates are also shown as there was as much variability between
758 experiments as within experiments), bars indicate medians. Statistics were performed using
759 the Mann Whitney test.

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

767

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

770 A, an automated *C. elegans* life-span machine was used to monitor the survival of worms in a
771 *P. aeruginosa*-mediated killing "slow killing" assay (McEwan et al., 2016). Approximately
772 200 wild-type *C. elegans* nematodes were fed WT PA14, or *ΔrhlI,* or *ΔrhlR PA14* mutants
773 (constructed in the Ausubel laboratory (red (*ΔrhlI)* and blue *(ΔrhlR)*) or Bassler (orange

774 (*ΔrhlI)* and green *(ΔrhlR)*) laboratories. P<0.001 (log-rank test) for PA14 vs *ΔrhlI,* PA14 vs
775 *ΔrhlR, or ΔrhlI vs ΔrhlR*. The experiment was repeated twice with similar results.

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

780 B and C. Representative images are shown.

781 D and E. Images were quantified using ImageJ. There was a significant difference in the
782 levels of live bacteria between WT PA14 and *ΔrhlR* (p = 0.034 (D) and 0.0097 (E)), and there
783 were significant differences between the *ΔrhlR* and *ΔrhlI* mutants (p = 0.0012 (D) and 0.0042
784 (E)), using a non-parametric Mann-Whitney test. The differences between *ΔrhlI* and WT
785 PA14 were not significant (p = 0.16 (D) and p = 0.95 (E)). The experiments were repeated at
786 least two times with similar results.

787

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

789 A and B, Wild-type *Drosophila* were orally infected with wild-type PA14 expressing dsRed
790 (PA14-dsRed). After 4 days infected flies were transferred to tubes containing wild-type
791 PA14 expressing GFP (PA14-GFP). At 5 days of the infection (one day after transferring flies
792 to PA14-GFP), most PA14 bacteria in the *Drosophila* gut expressed dsRed and only 10%
793 expressed GFP (A). However, at 6 days (2 days after the transfer of flies to PA14-GFP), only
794 GFP positive bacteria were detected in the gut. Results were similar for bacteria retrieved
795 from the hemolymph compartment (B).

796 C, D and E, LT_{50}s from survival experiments of WT flies (w) after intestinal infection with
797 PA14 WT (C), Δ*rhlR* (D) or Δ*rhlI* (E) mutants and injection of either latex beads (LXB, grey
798 dots) or PBS (black dots) at different time points of the infection. Latex beads or PBS were
799 injected either one day before the infection started (-1d) or four hours (+4h), one day (+1d),
800 four days (+4d) or six days (+6d) after the infection started. White dots correspond to the
801 survival of infected, uninjected flies. (C) LT50s of w^{A5001}-LxB are significantly lower than
802 w^{A5001} only at -1d (**p=0.0086) and +4h (*p=0.0154). (D) LT_{50}s of *w*-LXB flies are
803 significantly lower than *w* at most times during the infection (-1d: ***p=0.0002, +4h:
804 ***p=0.0002 and +4d: **p=0.0069). (E) A similar phenotype is observed with flies infected
805 with Δ*rhlI* (-1d: *p=0.0395, +4h: **p=0.0085, +1d: ***p=0.0002 and +4d: *p=0.0400). Note

806 however that for injections of latex beads at day4 the difference is reduced, as compared to
807 earlier time points of injection of latex-beads. The cumulative LT_{50} data from at least three
808 experiments (only two experiments for Δ*rhlI*) are shown, except for +6 d; bars indicate
809 medians. Statistical analyses were done with an unpaired t-test.

810 F. Differences of LT_{50}s between WT flies injected with PBS (WT) and flies injected with
811 latex-beads (WT^{LXB}) after intestinal infection with PA14 WT or Δ*rhlR* mutant or Δ*rhlI*
812 mutants in at least two experiments, bars indicate medians.

813 G. Guts of transgenic flies with GFP-labeled hemocytes were dissected in a manner that
814 preserves the association of hemocytes with the digestive tract and were examined by
815 fluorescence confocal microscopy. Green: GFP; blue: DAPI staining of nuclei. Scale bars:
816 100µM.

817 H. Analysis of hemocytes recruited to the fly intestine upon the infection with either wild-
818 type PA14, or Δ*rhlR* or Δ*rhlI* mutant bacteria. Both PA14 WT and the Δ*rhlR* or Δ*rhlI* mutant
819 bacteria induce a recruitment of hemocytes to the gut (4h after the beginning of the infection,
820 for each bacteria $p<0.0001$). At one day, there are slightly more hemocytes recruited when
821 infected with wild-type PA14 compared to the mutant. While at 3 days after the beginning of
822 the infection there are more hemocytes recruited when infected with the Δ*rhlR* mutant
823 bacteria ($p=0.0025$ between Δ*rhlR* and Δ*rhlI*). Data represent 3 pooled experiments. Bars
824 indicate medians.

825

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

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

830 A. *Tep4* and *Tep2,3,4* mutant flies are significantly more susceptible to PA14 infection
831 compared to w^{A5001}. No difference in survival was detected between the *Tep2,3* mutant and
832 w^{A5001}. Surprisingly *Tep3* mutants seemed to be more resistant to infection. B, A strong
833 enhancement of Δ*rhlR* virulence is observed with *Tep4* and *Tep2,3,4* mutants compared to
834 w^{A5001} flies. *Tep2,3* and w^{A5001} exhibited nearly the same rate of death when challenged with
835 Δ*rhlR* . The *Tep3* mutant seemed again to be more resistant to the infection. In A and B, one
836 representative experiment out of three (each with biological triplicates, except for uninfected
837 flies) is shown.

838 C. The survival of *Tep4* flies infected with PA14 WT or Δ*rhlR* or Δ*rhlI* bacteria was
839 examined. One representative experiment out of three (each with biological triplicates) is
840 shown.

841 D. Quantification of the experiments shown in C. The triplicates were assessed as
842 independent experiments as there was as much variability between experiments as within
843 experiments. p values comparing the LT_{50}s of mutants versus PA14 WT are from left to right:
844 0.07, **: 0.003, (Mann-Whitney test). Bars indicate medians.

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

848

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

850 A. Heat-killed pHrodo®-labeled bacteria of the indicated genotype were injected into either
851 wild-type or *Tep4* third instar larvae and incubated for 45 minutes. The hemocytes were then
852 retrieved. Bacteria present in phagosomes were fluorescent and used to measure the
853 phagocytic index. Bacteria injected into *Tep4* were significantly less phagocytosed than those
854 injected into wild-type larvae: p<0.0001 for PA14 WT and p=0.04 for Δ*rhlR* (Mann-Whitney
855 test). Bars indicate medians.

856 B. The experiment is similar to that shown in A, except that live bacteria were used and a
857 differential antibody staining protein was used to reveal phagocytosed bacteria. PA14 WT,
858 Δ*rhlR*, Δ*rhlI* bacteria were injected in wild-type or *Tep4* larvae. Δ*rhlR* bacteria were more
859 readily phagocytosed than PA14 WT both by wild-type (p=0.01) or *Tep4* (p=0.04) hemocytes
860 (Mann-Whitney test). Bars indicate medians.

861

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

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

866 B-E. Bacteria pre-incubated with wild-type or *Tep4* hemolymph are represented in pairs,
867 respectively with filled (left) and open (right) circles. Bars indicate medians. Data were
868 analyzed using the Mann-Whitney test. ***: p<0.0001

869 B. PA14 WT, Δ*rhlR*, and Δ*rhlI* bacteria pre-incubated with either wild-type (wt) or *Tep4*
870 hemolymph were injected into wild-type larvae. Stars above symbols report the statistical
871 significance of the data when compared to PA14 WT. **: p=0.007

872 D. PA14 WT, Δ*rhlR*, and Δ*rhlI* bacteria pre-incubated with either wild-type or *Tep4*
873 hemolymph were injected into *Tep4* larvae. **: p=0.01

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

876

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

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

880 C. *Diptericin* expression was measured by RT-qPCR in wild-type and *Tep4* flies, 2, 8 or 24h
881 after injection of PBS (NI), or 10, 100, 1000 cfus of PA14 WT bacteria. Each experiment was
882 performed independently three times and a representative experiment is shown (A-C). The
883 *Diptericin* expression shown is relative to *RpL32* expression and normalized to the
884 noninfected wild-type control.

885 D. The cleavage of the prophenol oxidase have been analyzed 2 hours after injection of 1000
886 cfus of PA14 WT bacteria or PBS, by Western-blotting using a pan-phenol oxidase antibody.
887 The intensity of the prophenol oxidase (PPO) and of the cleaved active phenol oxidase
888 (activated PO) bands was measured and the ratio of the measurements is shown below the
889 blot. This experiment was performed twice with a similar result.

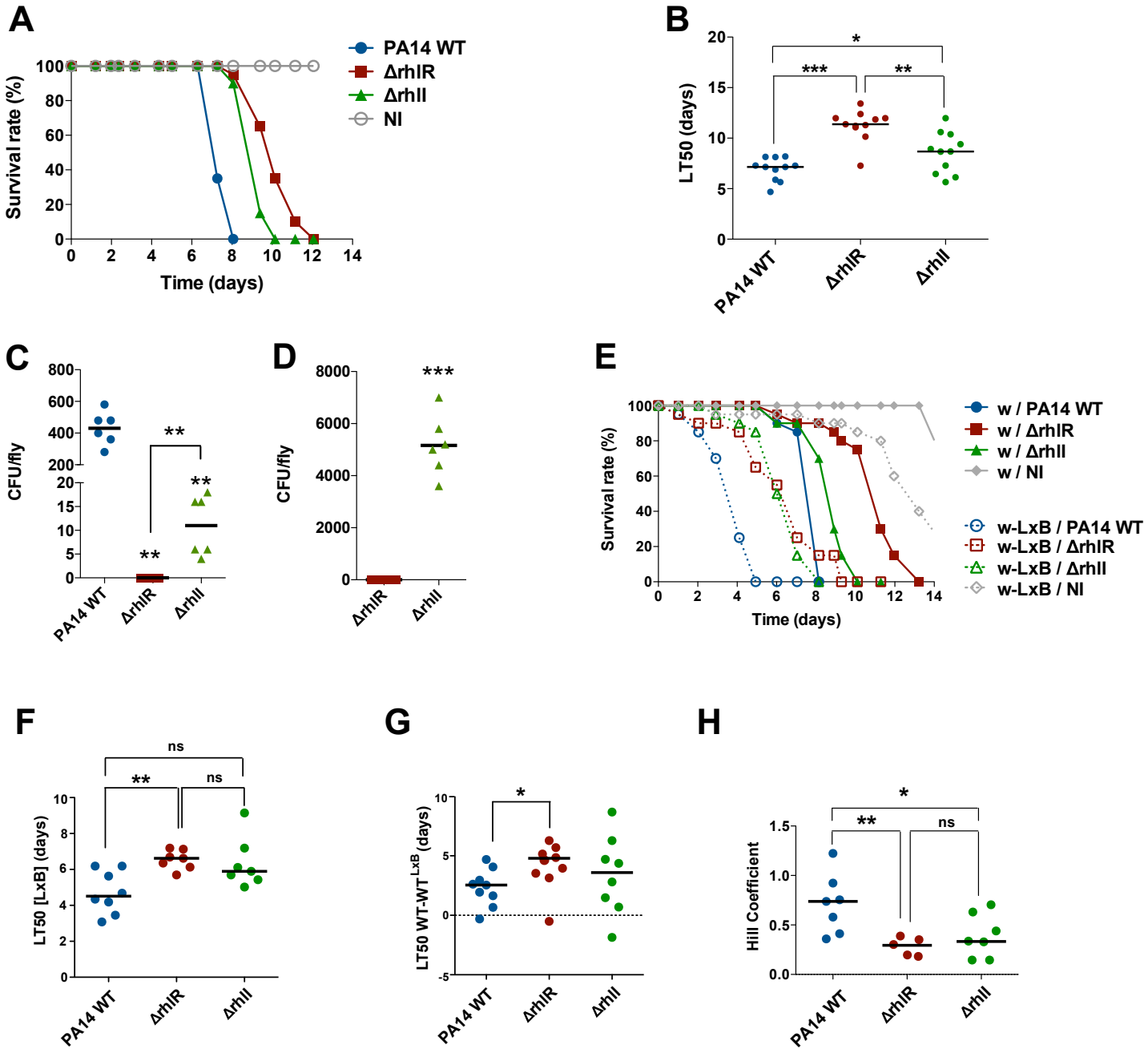
A
Survival rate (%)
Time (days)
PA14 WT
ΔrhlR
ΔrhlI
NI
B
LT50 (days)
PA14 WT
ΔrhlR
ΔrhlI
C
CFU/fly
D
CFU/fly
E
Survival rate (%)
Time (days)
w / PA14 WT
w / ΔrhlR
w / ΔrhlI
w / NI
w-LxB / PA14 WT
w-LxB / ΔrhlR
w-LxB / ΔrhlI
w-LxB / NI
F
LT50 [LxB] (days)
G
LT50 WT-WT^LxB (days)
H
Hill Coefficient

Figure 1

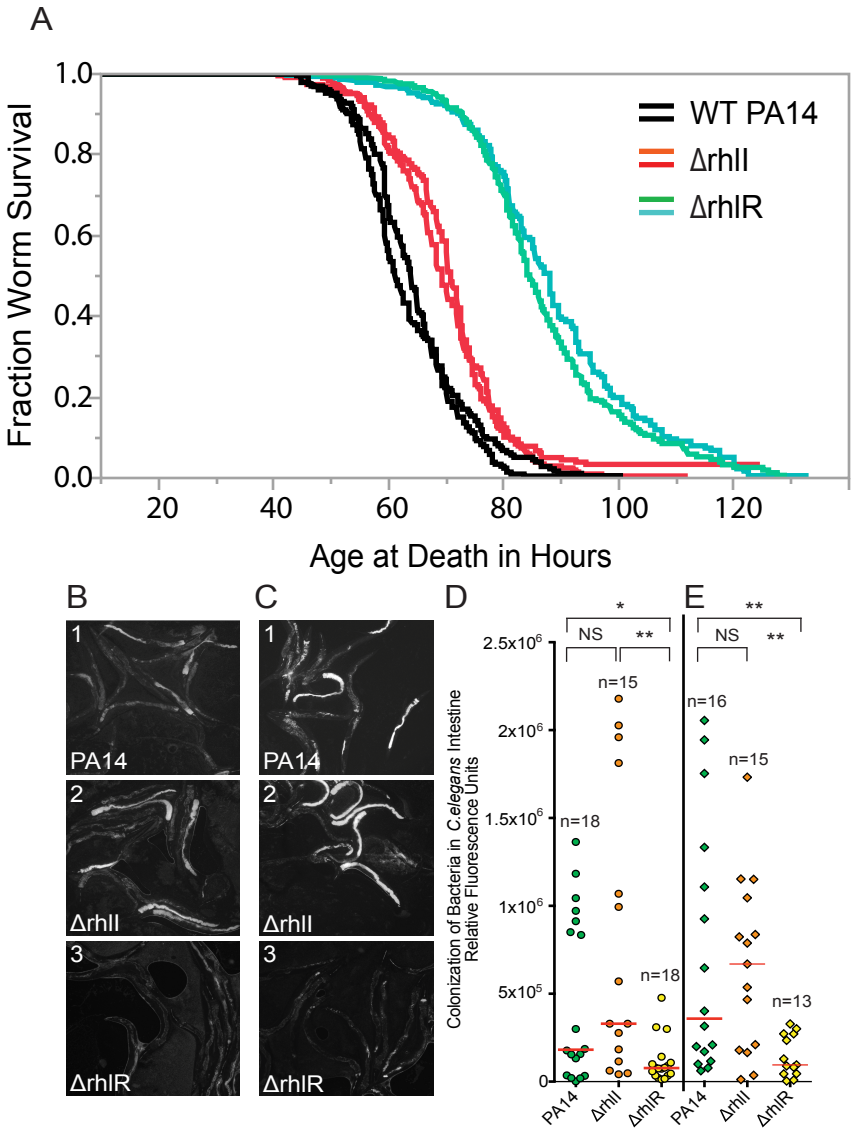
A
Fraction Worm Survival
1.0
0.8
0.6
0.4
0.2
0.0
20
40
60
80
100
120
Age at Death in Hours
WT PA14
ΔrhlI
ΔrhlR
B
C
D
E
1
PA14
2
ΔrhlI
3
ΔrhlR
Colonization of Bacteria in C.elegans Intestine
Relative Fluorescence Units
2.5x10^6
2x10^6
1.5x10^6
1x10^6
5x10^5
0
NS
**
*
n=18
n=15
n=18
n=16
n=15
n=13
PA14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

Figure 2

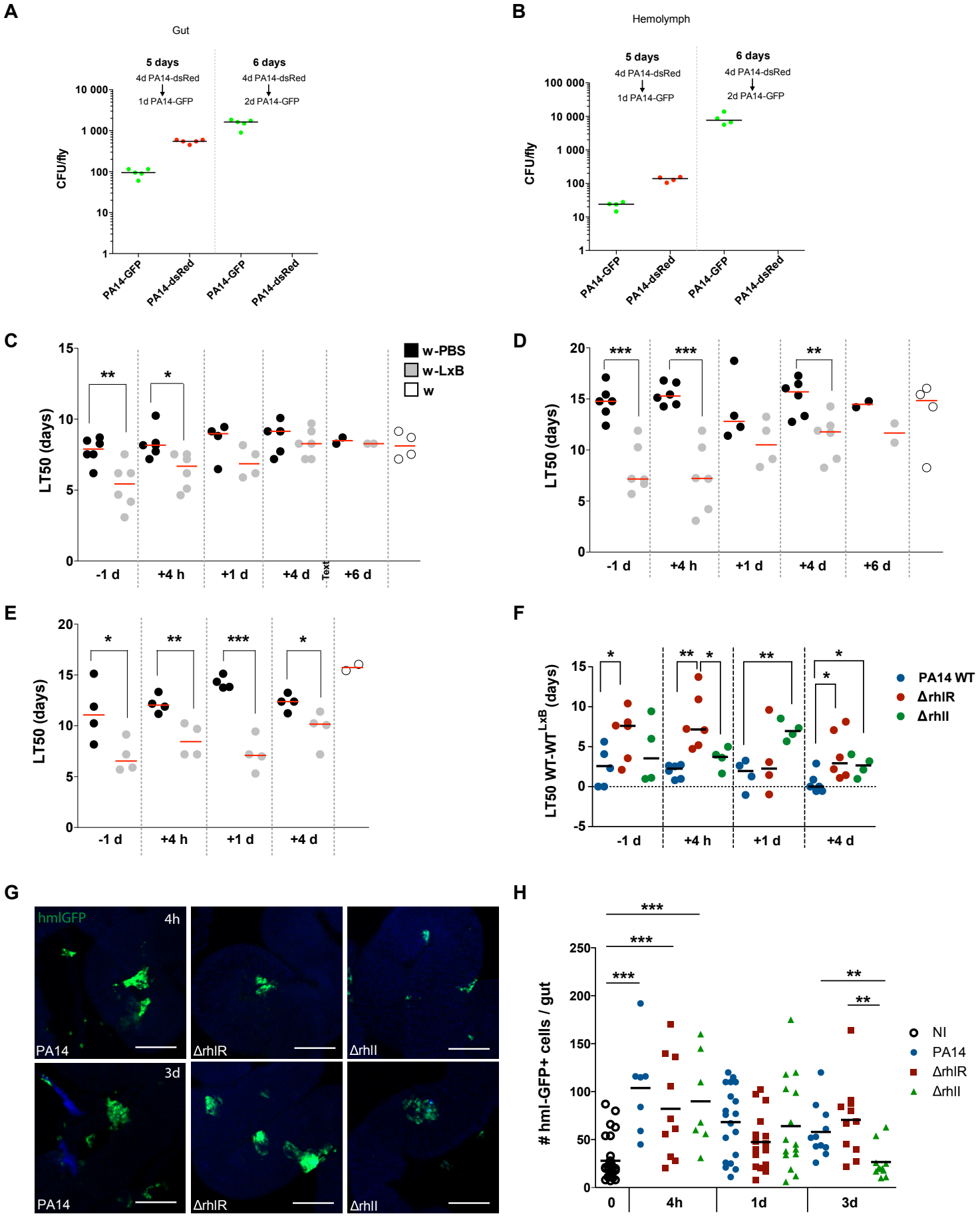
A
Gut
5 days
4d PA14-dsRed
1d PA14-GFP
6 days
4d PA14-dsRed
2d PA14-GFP
CFU/fly
10 000
1 000
100
10
1
PA14-GFP
PA14-dsRed
PA14-GFP
PA14-dsRed
B
Hemolymph
5 days
4d PA14-dsRed
1d PA14-GFP
6 days
4d PA14-dsRed
2d PA14-GFP
CFU/fly
100 000
10 000
1 000
100
10
1
PA14-GFP
PA14-dsRed
PA14-GFP
PA14-dsRed
C
w-PBS
w-LxB
w
LT50 (days)
15
10
5
0
**
*
-1 d
+4 h
+1 d
+4 d
Text
+6 d
D
LT50 (days)
20
15
10
5
0
***
***
**
-1 d
+4 h
+1 d
+4 d
+6 d
E
LT50 (days)
20
15
10
5
0
*
**
***
*
-1 d
+4 h
+1 d
+4 d
F
PA14 WT
ΔrhlR
ΔrhlI
LT50 WT-WT^LxB (days)
15
10
5
0
-5
*
**
*
**
*
*
-1 d
+4 h
+1 d
+4 d
G
hmlGFP
4h
PA14
ΔrhlR
ΔrhlI
3d
PA14
ΔrhlR
ΔrhlI
H
hml-GFP+ cells / gut
250
200
150
100
50
0
***
***
***
**
**
NI
PA14
ΔrhlR
ΔrhlI
0
4h
1d
3d

Figure 3

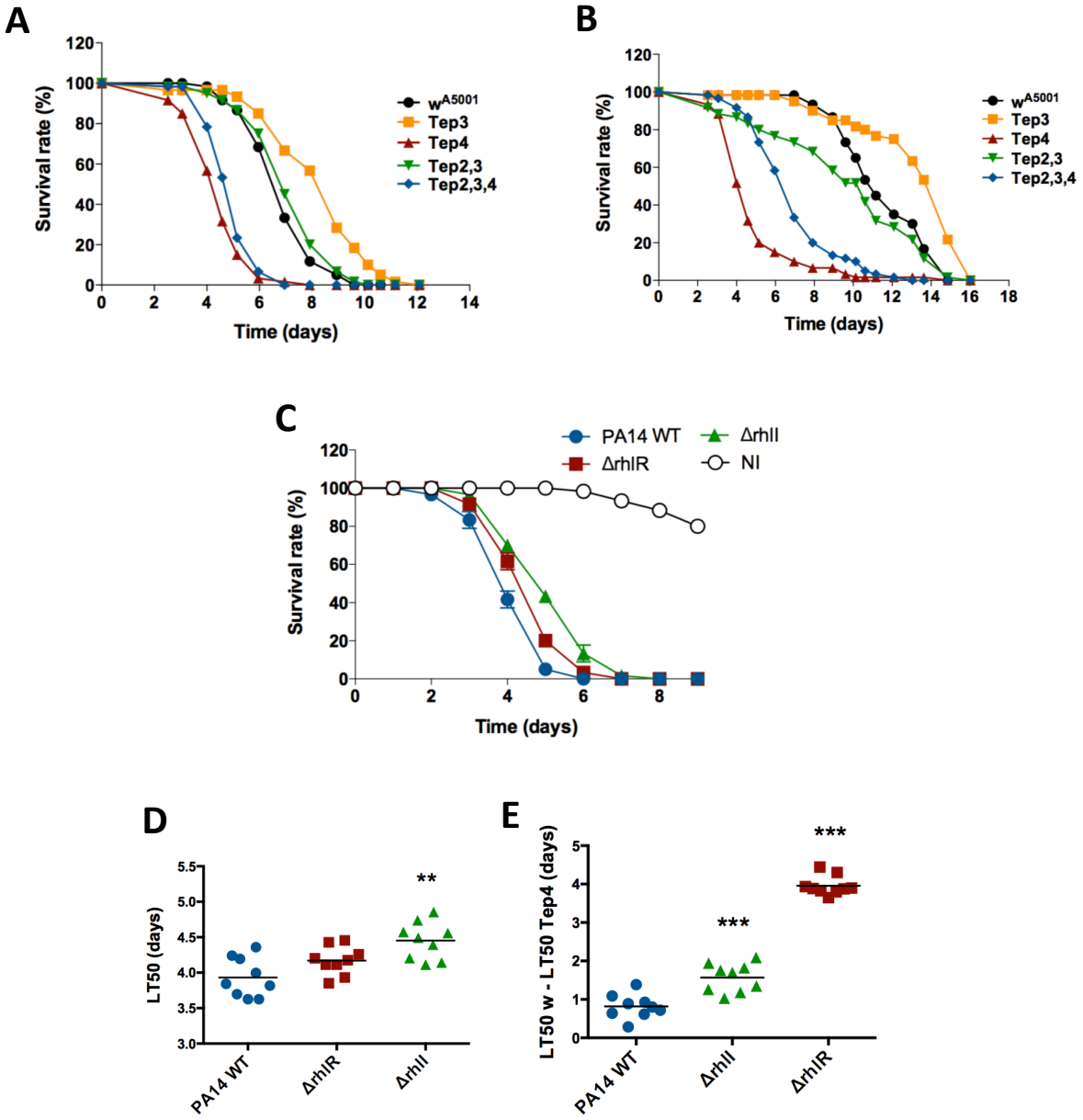
A
Survival rate (%)
Time (days)
wA5001
Tep3
Tep4
Tep2,3
Tep2,3,4
B
Survival rate (%)
Time (days)
wA5001
Tep3
Tep4
Tep2,3
Tep2,3,4
C
Survival rate (%)
Time (days)
PA14 WT
ΔrhlR
ΔrhlI
NI
D
LT50 (days)
**
PA14 WT
ΔrhlR
ΔrhlI
E
LT50 w - LT50 Tep4 (days)
***
***
PA14 WT
ΔrhlI
ΔrhlR

Figure 4

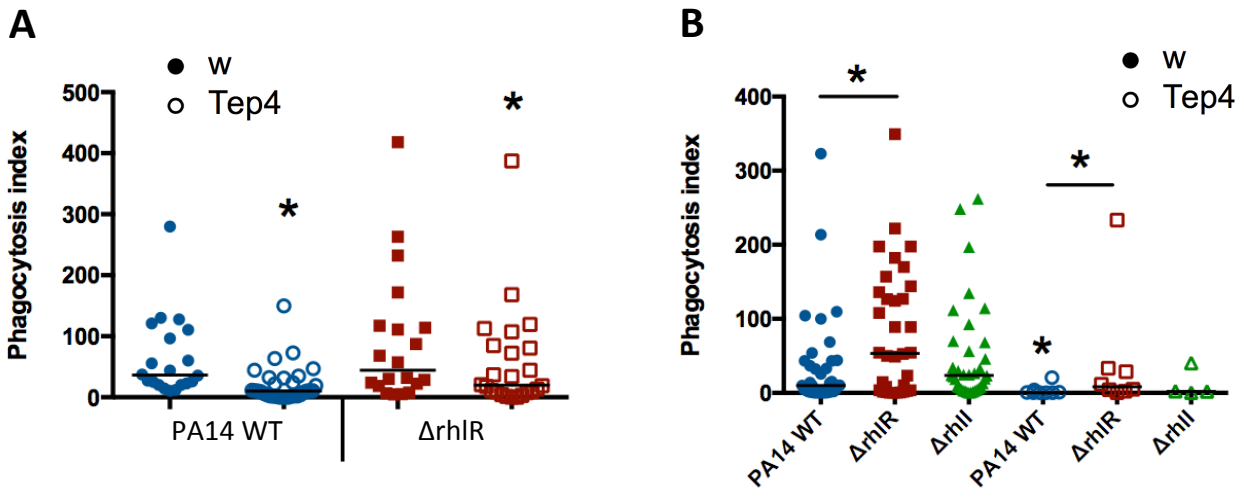
A
w
Tep4
Phagocytosis index
500
400
300
200
100
0
*
*
PA14 WT
ΔrhlR
B
w
Tep4
Phagocytosis index
400
300
200
100
0
*
*
*
PA14 WT
ΔrhlR
ΔrhlI
PA14 WT
ΔrhlR
ΔrhlI

Figure 5

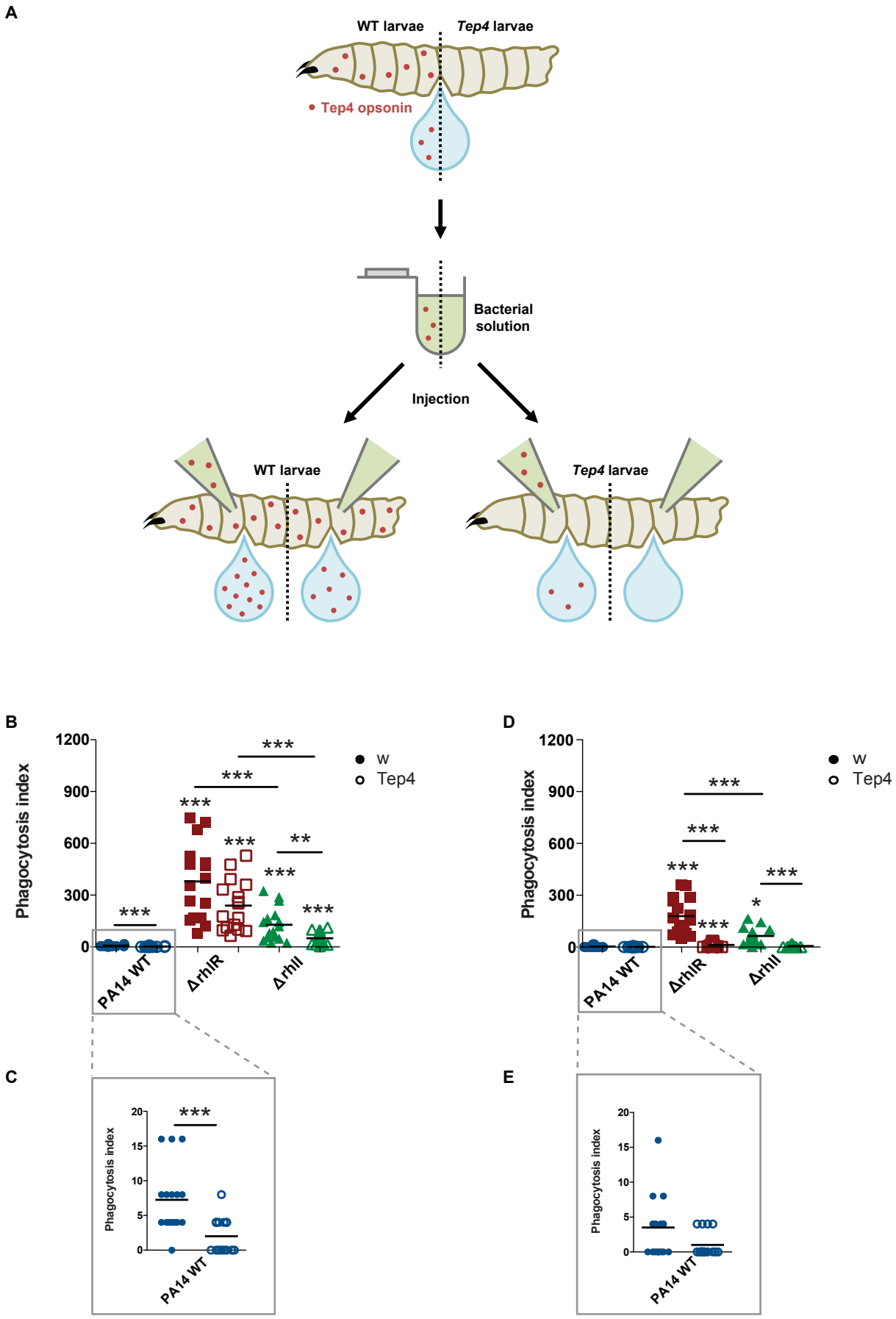
A
WT larvae
Tep4 larvae
Tep4 opsonin
Bacterial solution
Injection
WT larvae
Tep4 larvae
B
Phagocytosis index
1200
900
600
300
0
w
Tep4
PA14 WT
ΔrhlR
ΔrhlI
C
Phagocytosis index
20
15
10
5
0
PA14 WT
D
Phagocytosis index
1200
900
600
300
0
w
Tep4
PA14 WT
ΔrhlR
ΔrhlI
E
Phagocytosis index
20
15
10
5
0
PA14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

Figure 6

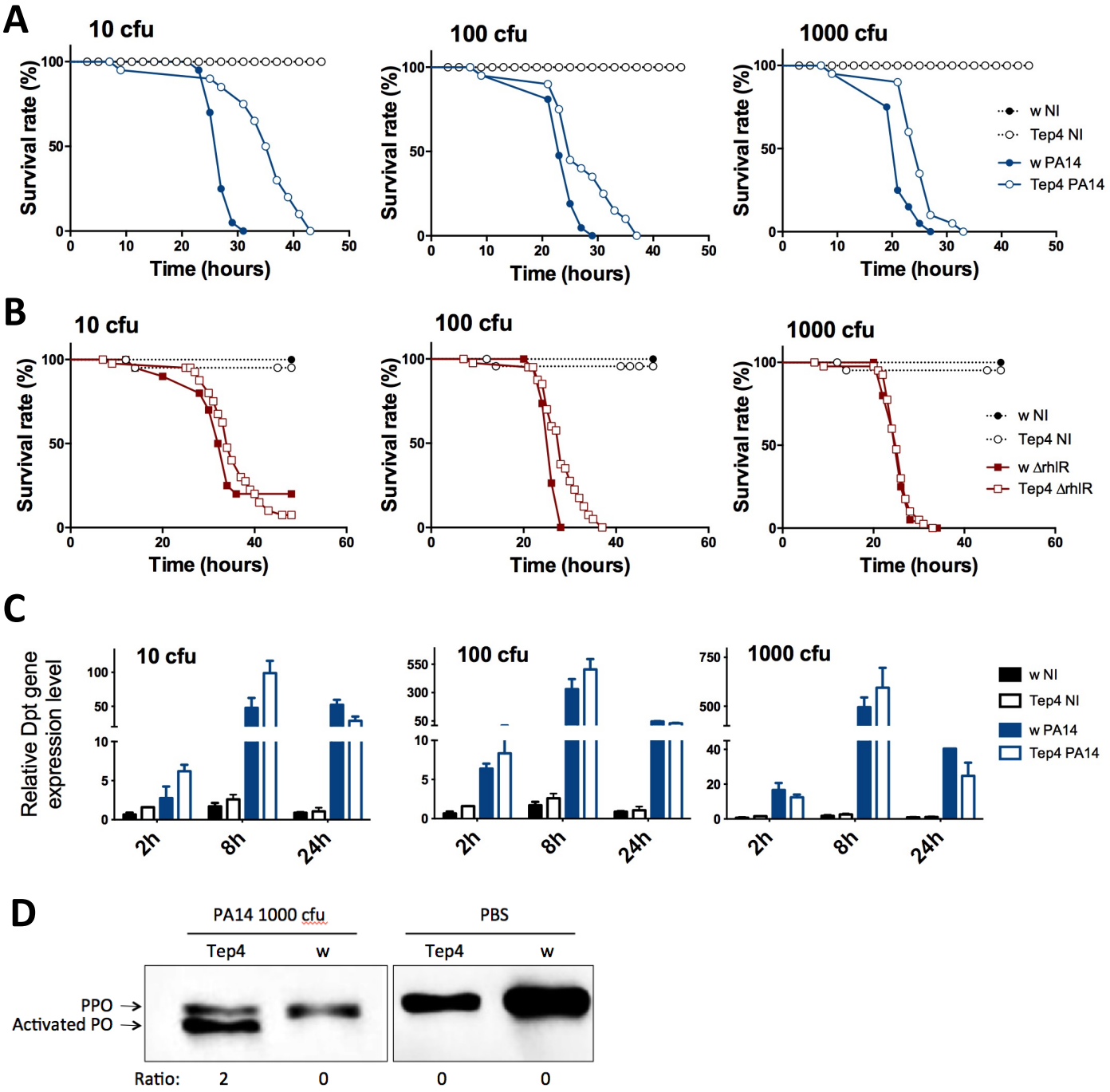
A
10 cfu
100 cfu
1000 cfu
Survival rate (%)
Time (hours)
w NI
Tep4 NI
w PA14
Tep4 PA14
B
w ΔrhlR
Tep4 ΔrhlR
C
Relative Dpt gene expression level
2h
8h
24h
D
PA14 1000 cfu
PBS
Tep4
w
PPO
Activated PO
Ratio:
2
0
0
0

Figure 7