

1 **Step-specific adaptation and trade-off over the course of an infection by**
2 **GASP-mutation small colony variants.**

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17 **Abstract**

18 During an infection, parasites face a succession of challenges, each decisive for disease outcome.
19 The diversity of challenges requires a series of parasite adaptations to successfully multiply and
20 transmit from host to host. Thus, the pathogen genotypes which succeed during one step might be
21 counter-selected in later stages of the infection. Using the bacteria *Xenorhabdus nematophila* and
22 adult *Drosophila melanogaster* as hosts, we showed that such step-specific adaptations, here linked
23 to GASP (i.e. Growth Advantage in Stationary Phase) mutations in the *X. nematophila* master gene
24 regulator *lrp*, exist and can trade-off with each other. We found that nonsense *lrp* mutations had
25 lowered ability to resist the host immune response, while all classes of mutations in *lrp* were
26 associated with a decrease in the ability to proliferate during early infection. We demonstrate that
27 reduced proliferation of *X. nematophila* best explains diminished virulence in this infection model.
28 Finally, decreased proliferation during the first step of infection is accompanied with improved
29 proliferation during late infection, suggesting a trade-off between the adaptations to each step. Step-
30 specific adaptations could play a crucial role in the chronic phase of infections in any diseases that
31 show similar small colony variants (also known as SCV) to *X. nematophila*. **Importance.** Within-
32 host evolution has been described in many bacterial diseases, and the genetic basis behind the
33 adaptations stimulated a lot of interest. Yet, the studied adaptations are generally focused on
34 antibiotic resistance, rarely on the adaptation to the environment given by the host, and the potential
35 trade-off hindering adaptations to each step of the infection are rarely considered. Those trade-offs
36 are key to understand intra-host evolution, and thus the dynamics of the infection. However, the
37 understanding of these trade-offs supposes a detailed study of host-pathogen interactions at each
38 step of the infection process, with for each step an adapted methodology. Using *Drosophila*
39 *melanogaster* as host and the bacteria *Xenorhabdus nematophila*, we investigated the bacterial
40 adaptations resulting from GASP mutations known to induce small colony variant (SCV) phenotype

41 positively selected within-the-host over the course of an infection, and the trade-off between step-
42 specific adaptations.

43 **Introduction**

44 Successful colonization of a host is essential to the lifecycle of pathogens. Over the course of an
45 infection, pathogens face a series of barriers to establishing an infection, each one decisive for the
46 outcome. Depending on the system and on our level of resolution, we can distinguish a variable
47 number of such steps. In the simplest case, parasites need to first encounter and attach to their host,
48 then to overcome the different lines of immune defence to proliferate and establish within the body,
49 and, finally, to transmit to another host (1–3). Each transition between two steps imposes a new
50 challenge that pathogens might overcome with step-specific adaptations. If traits involved in
51 different steps are uncorrelated, independent adaptative mutations may occur and increase in
52 frequency until the pathogen succeeds in each and every step of the infection. But those step-
53 specific adaptations may also trade-off with each other: the pathogenic strains which are successful
54 and dominant at the initiation of the disease might differ from those that are successful and
55 dominant at a given advanced step of an infection. Within-host evolution has been described in
56 many human bacterial diseases, such as *Helicobacter pylori* (4), *Staphilococcus aureus* (5), or the
57 causing agent of melioidosis - *Burkholderia pseudomallei* (6), and the genetic basis behind the
58 adaptations stimulated a lot of interest. However, the studied adaptations are generally focused on
59 antibiotic resistance, rarely on the adaptation to the environment given by the host, and the potential
60 trade-off hindering adaptations to each step of the infection, especially within the host, are rarely
61 considered (7).

62 Such trade-offs limit our understanding of how pathogens evolve until we precisely describe the
63 way they interact with their hosts at each step of the disease process. The bacteria *Salmonella*
64 *enterica* is a good example of model where trade-offs have been described (8). The *in vivo*
65 adaptation to a specific host trades off with the transmission to another host (9), and the adaptation

66 to remain in the blood trades off with the ability to infect the gastrointestinal environment by the
67 loss of key gene functions (10). In *Salmonella enterica*, phase variation (i.e. a mechanism for high
68 frequency back-and-forth switch between phenotypes) of the virulence factor impeding fast
69 proliferation over the course of the infection has been selected to avoid this trade-off (11), but this
70 mechanism is not present in all bacterial diseases and it is not clear whether bacterial adaptations for
71 each step are impeded by the trade-off.

72 The bacterial entomopathogen *Xenorhabdus nematophila* is a tractable model which can
73 help in understanding the consequences of step-specific adaptations on intra-host evolution. In the
74 wild, *X. nematophila* forms a symbiotic association with its vector, the nematode *Steinernema*
75 *carpocapsae*, which lives in soil and reproduces in insect hosts. Once in an insect gut, the vectors *S.*
76 *carpocapsae* inject a few cells of their bacterial symbiont into the insect haemolymph. The bacterial
77 population proliferates despite the host immune response, produces toxins to kill it rapidly, and
78 degrades host tissues, which in turn supports nematodes development and reproduction inside the
79 host body cavity. After the insect's death, the bacteria cannot disperse before the population of
80 nematode vectors grows. At this step of the infection, the bacteria and nematode must share the
81 dead insect as a stock of resources that are no longer replenished; nematodes then often eat the
82 bacteria to ensure their survival (12). Once dispersing nematode offspring are produced, a small
83 number of bacteria associate with them, thanks to a very specific set of genes, and disperse with the
84 vector (13–16). Thus, we can recognize at least three discrete steps in the infection (17): one where
85 the bacteria are in a dedicated vesicle of the nematode, waiting for the next infection; one where the
86 bacteria need to survive the insect immune response and destroy host tissue for the establishment of
87 its vector; and a final step where the bacteria need to persist in the decaying insect host to
88 eventually feed and colonise the vector.

89 For decades the entomopathogen bacteria *Xenorhabdus nematophila* has been described as
90 occurring under two phenotypes resulting from phase variation (18). Bacteria from the phenotype

often referred as 1 are mobile/flagellated, produce antibiotics, hemolysines, immune suppressors and toxins, while bacteria from phenotype 2 generally lack these characteristics (19, 20), and are about ten times smaller (7). However, it has recently been shown that those two discrete phenotypes are in fact not due to phenotypic switching but to selection during the infection of mutations in the Leucin-responsive regulatory protein (*lrp*) (7). Even though a variety of *lrp* mutations are known to be selected over the infection, the differences of advantage they could confer is unknown. *lrp* is a strongly conserved global transcriptional regulator widely distributed among prokaryotes and archaea (21–23). In *Escherichia coli*, it is involved in amino acid metabolism, monitors the general nutritional state by sensing the concentrations of leucine and alanine in the cell, and regulates genes involved in entering the stationary phase of growth (24, 25). In fact, *lrp* controls the gene expression of about a third of the genome (26) and acts as a virulence regulator in numerous infectious diseases, including those caused by *Salmonella enterica* serovar *Typhimurium* (27), *Vibrio cholera* (28, 29), *Mycobacteria* (30, 31), *Clostridium difficile* (32), and *Xenorhabdus nematophila* (33, 34). Comparing *in vitro* phenotypes linked to the presence or absence of the mutation in *lrp* with the cycle of the bacteria, one can make a clear hypothesis on how the mutants are selected over the course of the infection. The characteristics of the strains without mutation in *lrp*, described above, suggest that these bacteria are selected to invade a living host carrying other bacterial competitors that must be eliminated to prepare the environment for the nematode. Consistent with this hypothesis, in nature, nematodes generally carry a clonal bacterial population with phenotype 1, which we below name ‘wildtype strain’ (18). On the other hand, the typical characteristics of bacteria living in an environment limited in resources - stationary phase (35) - correspond to the characteristics of the bacteria in phenotype 2, below named ‘mutant’. This suggests that mutations in *lrp* could give an advantage to wait in a decaying host, where quality and/or quantity of resources or other conditions, such as pH, change. In fact, *lrp* mutants outcompete wildtype in aged *in vitro* cultures of *X. nematophila* (7), as does *Escherichia coli* (36), a

116 phenotype described as growth advantage in stationary phase (GASP) (37). Furthermore, *lrp*
117 mutants can outcompete wildtype *in vivo*, when they colonize mouse gut (38). Thus, a clonal
118 bacterial population selected to initiate the infection could be first favoured until the environment
119 changes, and mutants able to grow in stressful conditions become dominant. These mutants can
120 potentially associate with the nematodes, even though badly, but they have never been found in
121 wild-caught nematodes. However, they may serve as food or process available food to provide
122 nutrients for their vector until the rare wildtype genotype or a genotype with a compensatory
123 mutation, allowing phenotype reversion, can re-associate with the nematodes, disperse from the
124 host cadaver inside the new vector, and initiate a new infection. It is not clear whether acquiring this
125 adaptation to persist in the host would hamper the adaptation to initiate infection.

126 In this study we investigated the bacterial adaptations resulting from mutations known to be
127 positively selected within-the-host over the course of an infection and the trade-off between step-
128 specific adaptations. More specifically, we characterised the consequences of nonsense and
129 missense mutations in the major regulator gene *lrp* on *Drosophila melanogaster* infection at
130 different steps of the infections. Our results suggest that virulence decreased as mutations in *lrp*
131 become more disruptive to gene function, correlating with the ability to grow at the beginning of the
132 infection. Despite the fact that bacteria carrying nonsense mutations in *lrp* proliferated better in
133 immunodeficient compared to healthy hosts, the ability to cope with immune system activation did
134 not correlate with virulence. Furthermore, mutants killed hosts at similar bacterial loads to wildtype,
135 suggesting that they were as pathogenic as wildtype strains and that the ability to kill was solely
136 explained by the speed of proliferation within the host. Next, we demonstrated that mutants,
137 regardless of the type of mutation, were well-adapted to the waiting step of the infection as they
138 proliferated better in dead hosts than do wildtype. Our results suggest that wildtype strains perform
139 better than mutants during the first step of infection, but less well during the second step. We
140 demonstrated that as the infection progressed, the ability to grow well in dead host was acquired

141 while the ability to grow well in the healthy host was lost, suggesting a trade-off between the
142 adaptations to each step.

143 **Results**

144 **The bacterium-host model.**

145 *Xenorhabdus nematophila* is a generalist, and highly virulent entomopathogen bacterium. It is
146 generally described as having two distinct phenotypes, distinguished in the lab by their capacity to
147 adsorb a dye (bromothymol blue). This phenotype is associated with mutations in the *lrp* gene
148 occurring *in vitro* and *in vivo*, which the wildtype strains do not carry. The strains used in this study
149 were chosen from a collection of 34 strains; some are wildtype for the *lrp* gene (blue color in the
150 following results), while the others carry various classes of mutation in different domains of *lrp* (red
151 in the following results). *D. melanogaster* is not a natural host of our generalist parasite, but the
152 genetic tools this model offers allowed us to test predictions on the role of arthropod host immunity
153 in the success of wildtype and mutant bacteria (39–41).

154 ***lrp* mutations impede virulence.**

155 We investigated the difference in virulence between *X. nematophila* strains. Using Canton S
156 wildtypes as host genotype, we found that all strains killed their host in less than two days post-
157 injection, but flies infected with wildtype bacteria died more rapidly than those infected with
158 mutants (Figure 2A; Coxme, $\chi^2=21.4$, $p=3.7e^{-06}$). Where wildtype bacteria killed on average 50 %
159 of their hosts in approximately 14 hours, mutants killed almost none of their host (~7 %). We
160 computed hazard ratio relative to sham infection (Figure 2B): the risk of death was increased on
161 average by a factor of $10^{3.3}$ when hosts were infected with wildtype strains, but only by a factor of
162 $10^{2.3}$ while infected by strains with a missense mutation in *lrp* or $10^{1.8}$ with strains with a nonsense
163 mutation.

164 **Wildtype strains grow faster at the beginning of the infection than *lrp* mutants.**

165 We hypothesized that if wildtype strains are adapted to initiate the infection, they should grow
166 faster than *lrp* mutants in the early stages of the infection. To test this hypothesis, we compared the
167 bacterial load of hosts infected with four wildtype and four *lrp* mutant *X. nematophila* strains, eight
168 hours post-injection. Overall, hosts infected with wildtype strains had higher bacterial loads 8 hours
169 post-injection (fitme *Mutation* effect: df=1, LRT= 153.39, p-value= 3.13e⁻³⁵, Figure 3A). After only
170 8 hours of infection, strains with a nonsense mutation in *lrp* were already four divisions or more
171 (i.e. four unit in log₂) behind other strains (medians log₂ bacterial load: nonsense mutants:
172 #36=11.5 and #40=11.6; other strains: #23=18, #42=17.2, #44=15.5, #51=15.7, #25=14.6,
173 #29=15.1, Figure 3A). Only mutant strain 29, bearing a missense mutation, had non significantly
174 lower load than wildtype strains after 8 hours of infection. We tested if the difference in bacterial
175 load between wildtype and mutant strains was linked, at least in part, to the host immune response.
176 As a Gram-negative bacterium, the main immune pathway activated by *X. nematophila* infection in
177 *Drosophila* is the IMD pathway. The IMD pathway is so important in controlling Gram negative
178 infections that individuals lacking the pathway can die in few hours from infections that are
179 otherwise benign (42, 43). It is already known that *X. nematophila* triggers a sustainable IMD
180 response upon systemic infection, that immune deficient hosts die earlier, and that hosts immune
181 primed by other Gram-negative bacteria survive longer to subsequent infection by *X. nematophila*
182 (39, 40). Thus, we compared the bacterial load in control healthy hosts to hosts carrying a knock-
183 out mutation in *Dredd*, a critical gene for the activation of the IMD pathway (44), at 8 hours post-
184 injection. We found that bacterial strains with a nonsense *lrp* mutation had a significantly higher
185 bacterial load in immunodeficient hosts than in healthy hosts, while wildtype and missense bacterial
186 strains had the same bacterial load (Figure 3A). To further support the result suggesting that
187 nonsense mutations in the *lrp* gene trigger a susceptibility to the host immune system, to which
188 wildtype strains seem to be fairly insensitive, we compared the difference in growth between 0 hour

189 and 8 hours post-injection (Figure 3B). The impact of IMD expression on bacterial proliferation can
190 be quantified as an interaction term in a statistical analysis of bacterial load which includes host
191 genotype (healthy vs immunodeficient) and time (0 vs 8h) as explanatory factors. We found that the
192 interaction was strongly significant only for one of the two nonsense mutations (strain 40, Figure
193 3B). However, even if not significant when tested overall, we found a lower proliferation for the
194 other strain with a nonsense mutation (#36) in two out of three replicates (Figure 3B). The
195 virulence, expressed as a higher hazard ratio, correlated with the ability to grow at the beginning of
196 the infection (Figure 3C). However, even if nonsense mutations proliferated better in
197 immunodeficient than in healthy hosts (Figure 3D), the ability to proliferate with the immune
198 system, as expressed as the difference in proliferation in healthy and immunodeficient hosts (i.e. the
199 statistical interaction mentioned above), did not correlate with the virulence (Figure 3E).

200 ***lrp* mutations do not alter bacterial pathogenicity.**

201 The mutations could change virulence via a change in bacterial pathogenicity (i.e. the ability
202 to kill at a given load). We investigated the role of the mutation on pathogenicity by estimating the
203 maximal bacterial load a host can sustain before dying (i.e the Bacterial Load Upon Death or
204 BLUD, see (2)). For a given host genotype, with a fixed tolerance, the variation in BLUD when
205 infected with different bacterial strains reflect the different amounts of damages these strains cause
206 to their host. In that case, the BLUD can thus be considered as a proxy measure of pathogenicity.
207 We hypothesized that the *X. nematophila* wildtype strains, which kill faster, would be more
208 pathogenic (e.g. secrete more toxins) than the mutants and thus, that hosts would succumb to a
209 lower load with wildtype strains than with mutant strains; i.e. the BLUD would be lower in hosts
210 infected by wildtype strains. We found that even if hosts died earlier from infections with wildtype
211 strains than with *lrp* mutant strains, the BLUD was the same (fitme, df=1, LRT = 2.51, p-
212 value=0.11, Figure 4A). As even nonsense mutations had the same BLUD as the wildtype, it is
213 likely that *lrp* does not have a role in the pathogenicity of *Xenorhabdus*. This is further suggested

214 by the absence of correlation between the mean of the hazard ratio of a strain, a proxy for its
215 virulence, and its BLUD mean (Figure 4B) and by previous results showing that *lrp* mutation does
216 not abolish *in vitro* insecticidal secretion activity (39).

217 ***lrp* mutants are better at proliferating in the dead host.**

218 We investigated the difference in growth in dead hosts by first estimating the BLUD and then the
219 load 24 hours later. Assuming that *lrp* mutants are selected by the new environment associated with
220 the host death, we hypothesized that they would proliferate better during these 24 hours. All strains
221 were able to grow in dead hosts, however, mutants had a much better ability to grow in those
222 conditions than did wildtype strains (Figure 5A). Interestingly, carrying a missense mutation in *lrp*
223 was sufficient for a strain to be better adapted to this condition, growing at a comparable rate to
224 strains carrying nonsense mutations (Figure 5B).

225 **Trade-off of step-specific adaptations due to *lrp* mutations.**

226 There was a clear qualitative trade-off between the ability to proliferate early in the infection and
227 the ability to proliferate in the dead host (Figure 5B & 6). The type of mutation did not determine
228 this trade-off and, even if there was a strong trend, we could not quantitatively show a negative
229 correlation between those two abilities (Figure 6, rho= -0.67, df= 6, t= -2.2, p=0.06). This suggests
230 that changes to, or abrogation of, Lrp function improves performances during the waiting step of the
231 infection but reduces drastically performances at the initiation of the infection.

232 **Discussion**

233 At each step of an infection, infected individuals can potentially stop the progress of their parasites.
234 Consequently, there is strong selection on parasites to reach the next step and complete their life
235 cycle (3, 45). However, the traits allowing to progress through each step may be strikingly different
236 and sometimes even be mutually costly. Using *D. melanogaster* as host and *Xenorhabdus*

237 *nematophila* as a bacterial pathogen, we showed that indeed the bacterial physiological
238 requirements for successive steps of infection are different and can trade-off.

239 In the system *Xenorhabdus nematophila* (bacteria) – *Steneirnema carpocapseae* (nematode)
240 – insect, the nematode vector injects the bacteria in the insect host body cavity to kill it, and then
241 uses the nutrients of the dead host to reproduce. At the beginning of the infection, bacteria
242 proliferate in a given environment where they need to be highly virulent to prepare the conditions
243 for their vector; once the host is dead, the environment necessarily changes (e.g. in terms of
244 nutrients, oxygen concentration or pH level) and bacteria need to optimize their use of resources
245 that are not replenished until the nematodes produce dispersing larvae. Part of this optimization is
246 accomplished by gene regulation. But over the course of an infection, mutations also occur in *X.*
247 *nematophila* populations. The mutations in *lrp* increase in frequency in the population (7), but if
248 they potentially can be carried by the vector, they re-associate poorly (16, 39), possibly because *lrp*
249 affects the expression of genes involved in the mutualism (46). This suggests that wildtype strains,
250 which are those found in wild-caught founder nematodes (18, 47), are adapted to disperse and
251 initiate the infection, while mutants are adapted to persist in the host. We found that both wildtypes
252 and *lrp* mutants proliferated faster *in vivo* at the step they were expected to be better adapted.

253 The control of bacterial proliferation by *Drosophila melanogaster* immune system can take
254 several hours (2) and the acquired immune system has evolved to reduce this crucial time where
255 bacteria proliferate exponentially if unintended. Hence, to increase their chance for a successful
256 infection, bacteria can adapt to either suppress, resist, or outpace the humoral immune response (i.e.
257 the control via antimicrobial peptide, AMP, secretion) by killing the host before the AMP
258 concentration is high enough to control its proliferation. Our results show that wildtype strains are
259 most likely better adapted to start the infection because 1- they proliferate faster in the first 8 hours
260 of the infection, 2- they do so at the same speed with or without the host immune response, unlike
261 most *lrp* mutants, and 3- they kill hosts at the same load as *lrp* mutant strains, but do so ten hours

earlier. The way in which wildtype bacteria are better adapted to the first step of the infection is still not entirely clear. The efficacy of *Drosophila* cellular immunity in adult is likely to depend on the infection and is regularly weak (2, 48). Therefore, *Drosophila* might not have a sufficiently strong cellular immune system to control a virulent pathogen such as *X. nematophila*, unlike the cellular immunity of Lepidopteran. For this reason, our study may under-estimates the potential adaptation of *X. nematophila* to cellular immunity. Yet, when the *Drosophila* humoral immune response is activated by a pre-exposure to an avirulent bacteria, *X. nematophila* is sensitive to the circulating AMPs (39, 40). This suggests that AMP can control the infection and that *X. nematophila* is not specifically resistant. However, *X. nematophila* is known to not only immunosuppress cellular immunity in Lepidopteran larvae (49, 50) but also downregulate *cecropin*, an AMP (51). Hence, it is likely that wildtype bacteria delay the humoral immune response to kill the host faster. However, *lrp* mutants still kill their host relatively quickly and the ability to grow despite the immune response did not correlate quantitatively with death hazard ratio, which suggests that the efficiency to immunosuppress the host is not the main reason for the higher virulence of the wildtype. However, the death hazard ratio correlated with the speed of proliferation during the first step of infection, which suggest that the adaptation to this step is mainly the intrinsic proliferation rate in this environment.

Although the mutants proliferate in larger numbers in dead hosts showing that they are better adapted to the second step of infection, they re-associate poorly with the nematode (39) and in addition, this adaptation traded-off with the ability to grow at the first step of the infection. If they are not found in wild-caught nematode and are not good at initiating an infection, it is not trivial to understand how mutants are so prevalent in the system. The *lrp* mutants could be selected inside the body of their host, during the step of proliferation, but would not have an advantage over the whole transmission cycle, as they do not favour a good transmission. This phenomenon is reminiscent of the short-sighted selection of cancerous cells within their host. However, because

287 wildtypes with mutant offspring would have a lower reproductive success, it is likely that any
288 mechanisms preventing those mutations would be advantageous. Alternatively, the occurrence of
289 the mutants may still be important in the symbiosis if they give an advantage to their kin wildtypes.
290 Indeed, *lrp* mutants are likely to modify the nutritional value provided by a dead host and, because
291 they can grow in higher numbers in the dead host, they can feed the vector by being preys. Hence,
292 by feeding the vectors directly or indirectly, the mutants may sustain the system and allow the
293 nematode to disperse with a kin wildtype strain or with a mutant which would have reverted its
294 phenotype.

295 In our system, the step transition between persistence in a living host and in a dead host is an
296 illustration of the different transitions which can happen in many other diseases, and how trade-off
297 between step-specific adaptations can occur. The change occurring between the acute phase of an
298 infection and its chronic phase is similarly one important and common transition over the course of
299 an infection. One of the bacterial adaptations to this transition is known as small-colony variants
300 (SCV). SCV is important for the chronic establishment of many human diseases such as for
301 instance *Staphilococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella* serovar, *Vibrio cholerae*,
302 *Brucella melitensis*, *Shigella* spp. or *Neisseria gonorrhoeae* (reviewed in Proctor et al., 2006). The
303 pathogen *Staphilococcus aureus* is a good example to illustrate the selection intra-host of SCV as it
304 is its ability to change rapidly from an extracellular aggressive state to SCV adapted for intracellular
305 infection, which allows for long-term persistence in its host. The typical *S. aureus* SCV form small
306 colonies on agar plates, have a decreased respiration, decreased pigmentation, decreased hemolytic
307 activity, decreased coagulase activity, increased resistance to aminoglycoside, and unstable colony
308 phenotype (52). *In vivo*, SCV usually gain in fitness by acquiring the ability to establish and remain
309 chronic in the host after they went through the acute phase of the infection. The phenotype of *X.*
310 *nematophila* which is selected during the waiting step (i.e. that of the mutant strains of our study) is
311 very similar to the commonly described SCV (7, 19). In fact, SCV was observed in *X. nematophila*

312 but not identified as such (53) and the term has been used to describe the alternative phenotype of a
313 closely related species, *Photorhabdus luminescens*, which also needs to wait in the dead insect until
314 its nematode vectors disperse (54–56). The biochemical basis of SCV in mammalian pathogens is
315 generally a single or multiple auxotrophism caused by a simple genetic alteration, exemplified by
316 mutations of genes involved in the biosynthesis of thiamine, menadione, hemin or thymidine (57).
317 Mutations in *lrp* have indeed been found to be involved with induced auxotrophy in *E. coli* and
318 *Komagataeibacter europaeus* (58, 59) and with SCV phenotype in *Pseudomonas aeruginosa* (60).
319 Likewise, mutation in *lrp* is sufficient to switch to SCV-like phenotypes in *X. nematophila*,
320 allowing it to persist in the dead host until the vector disperses. Hence, we believe that our bacteria-
321 insect model system allowed us to study the advantage that SCV can provide over the course of an
322 infection and the trade-off between adaptations of infection steps. As such, it suggests that bacteria
323 initiating a disease can be drastically different from the bacteria selected within the host at later
324 stages of infection, and that intra-host selection is a factor to take into account to understand a
325 pathogen and its weakness during an infection.

326 Materials and methods

327 Fly stocks and husbandry

328 *Drosophila melanogaster* were reared on flour-yeast medium (per liter of water: 70 g of corn flour,
329 70 g of yeast, 15 g of agar, 10 g of vitamins (Vanderzant vitamin mixture for insects from Sigma-
330 Aldrich), 10 g of tegosept -diluted in 20 ml of ethanol- and 3 g of propionic acid). Males between
331 three and nine days old (age was standardized within each experiment) were used in all
332 experiments. Rearing and experiments were conducted at 25°C ($\pm 1^\circ\text{C}$) with a 12 h/12 h light/dark
333 cycle. We used Canton S flies and *w¹¹¹⁸* inbred, laboratory strains as wildtype genotypes. To test for
334 the susceptibility of the bacteria to the immune system, we used an immune-deficient host line in
335 which Imd pathway signalling is impaired due to a mutation in the gene *Dredd^{D55}* (61). The levels
336 of antimicrobial peptides in response to infection of *Dredd^{D55}* flies, backcrossed in the *w¹¹¹⁸*

337 genotype, has not been quantified in our study but has been shown as much lower in several
338 previously ones (e.g. (62)). This *Dredd* allele has even been confirmed recently by resequencing. It
339 is at X:634,862. A G to A nucleotide substitution alters the codon from TGG (Tryptophan, W) to
340 TAG (STOP) which produces a truncated protein (Personal communication to FlyBase
341 #FBref0243539 by François Leulier).

342 The bacterium

343 *Xenorhabdus nematophila* is generally described as having two distinct phenotypes, distinguished
344 by their capacity to adsorb bromothymol blue. When plated on a bromothymol agar (NBTA)
345 nutrient (15 g of Nutrient agar, 3 g of beef extract, 5 g of peptone, 8 g of NaCL, 0.04 g of Triphenyl
346 2,3,5 tetrazolium chloride (TTC) and 25 mg of bromothymol blue per liter of water) wildtype
347 bacteria form blue colonies while others form red colonies. This phenotype is associated in
348 mutations in the *lrp* gene, which the wildtype strains do not carry (7, 19, 34).

349 Our strains were chosen from a collection of 34 strains isolated from independent *in vitro* culture
350 after several days of a prolonged non-agitated growth in LB medium (Luria Broth, Miller) (see
351 their description in (7)). Although this well characterized collection has been obtained from *in vitro*
352 culture, similar mutants have been found *in vivo* (7). Strains 21, 23, 42 ,44, 51 are wildtype for the
353 gene *lrp*. *Lrp* is composed of two domains; a DNA-binding domain called HTH, and a ligand-
354 binding domain called RAM (63) The latter generally interacts directly with amino acids while the
355 former, in response to amino acid concentration, interacts with DNA to modify the expression of
356 hundreds of genes (21, 22). The strains 25 and 29 have a missense mutation (i.e. a single base pair
357 change in the domain RAM of *lrp*, both in codon position 120 - in SNP 358 and 359, respectively -
358 leading to amino acid substitutions). Strains 36, 39 and 40 have an indel mutation in *lrp*. The first
359 two have a frame shift in the HTH domain (in codon position 53), while 40 has a nonsense mutation
360 (i.e. 26 bp insertion in SNP 372 leading to a stop codon) in the RAM domain. Hence, while
361 mutations in strains 25 and 29 are expected to affect the function of the protein, mutations in the

362 strains 36, 39 and 40 are expected to stop its function completely (Details are also summarized in
363 Figure 1). To summarise, we used two strains of *X. nematophila* per mutation type, such that our
364 results will not be particular of one genotype.

365 **Quantification of bacterial suspension**

366 *X. nematophila* was grown in liquid LB medium. Overnight cultures, started from a single bacterial
367 colony, were grown to saturation at 28°C in agitated conditions (180 rpm). To prepare the
368 suspension used for injection, we first centrifuged the culture (10000 rpm for 5 min), discarded the
369 supernatant and re-suspended the bacterial pellet in 1 ml of LB, measured optical density (OD₆₀₀) by
370 spectrophotometer, and adjusted by dilution to an OD₆₀₀ of 0.1. To standardize the quantities of
371 bacteria injected inside the host, and because wildtypes and mutants differ in their absorbance, we
372 also used a Thoma cell counting chamber to enumerate cells under a microscope (Olympus BX 51,
373 magnification x200).

374 ***Xenorhabdus nematophila* injection in *Drosophila melanogaster***

375 We injected approximately 1000 bacteria per fly in 23 nl of LB medium, between the two first
376 abdominal segments using a Nanoject 2 injection system (Drummond) (64). Controls were injected
377 with 23 nl of sterile LB medium. Prior to injection, flies were anesthetized with CO₂. Anesthesia
378 lasted for about five minutes, and flies were observed afterward to confirm they recovered from the
379 procedure.

380 **Host survival**

381 Upon injection of approximatively 1000 bacteria, ~50 flies per replicate were kept in 900mL plastic
382 boxes at 25°C with 60 % humidity, and with *ad libitum* access to food. Survival was scored hourly,
383 starting around 10h post-injection. The dose of bacteria injected was chosen with the rationale that
384 it was within a realistic range for initial load with respect to natural infections, but high enough to
385 be sure that each host was exposed to the bacteria, as very low doses are more prone to random

386 variation in bacterial number, with in some cases no bacteria being injected. All flies died rapidly
387 upon injection, showing that they were all exposed.

388 **Estimation of bacterial load**

389 To characterize the bacterial within-host dynamics, we quantified the bacterial load in individual
390 flies at two time points during the infection. To extract bacteria from the host, we homogenized
391 individual flies at 30 hertz for 1 minute using a tissue lyser (Tissue Lyser II) in 250 µl of LB
392 medium containing two 2 mm glass beads. At least eight independent replicate measurements (*i.e.*
393 separate extractions on 8 individual flies) were performed per time point, treatment, and
394 experiment. Samples were serially diluted 1 to 1:2500 in 96-well microplates. We then deposited 5
395 µl drops from each well onto a NBTA plate using a 96 micropipette Integra Viaflo 96. Plates were
396 incubated for 48 hours at 28°C and we then counted the number of colonies that grew within each
397 drop. Such raw data were used in the analysis, but for graphical display we rather used estimations
398 of bacterial loads per fly obtained by adjusting a Poisson generalized linear model where the
399 number of CFU is predicted from dilution. To estimate the Bacterial Load Upon Death (BLUD) (2),
400 infected hosts were checked every 30 minutes and newly dead flies were collected and
401 homogenized, with bacterial load quantified as described above (2). Bacterial load 24 hours after
402 the host death was quantified from individuals kept in closed sterile microtubes. To estimate “Early
403 growth in wildtype” and “Growth post death” we extracted the estimates of the effect of the variable
404 “time”, and their standard error, from the models used to analyse the growth of each strain. To
405 estimate “Early growth in wildtype compared to immunodeficient” we extracted the estimates of the
406 interactions between “time” and “*D. melanogaster* lines” from the model used to analyse the
407 difference in growth in control line versus immunodeficient line of each strain.

408 **Statistical analyses**

409 We carried out all analyses using R (65). We analysed differences in survival (time-to-death curves)
410 using the packages “Survival” and “coxme” (66). We used the *coxme* routine, which allows

411 inclusion of random effects in a Cox model, to model variability among day of experiments and
412 replicated vials. We determined how host survival is affected by bacterial mutations by comparing a
413 Cox model including mutation status as a fixed effect to a model without this fixed effect. We
414 extracted hazard ratio from this survival model to compare survival among strains, taking into
415 account experimental replications. We analysed the differences in bacterial load within the host
416 using general linear mixed models (GLMM) implemented in the package *spaMM* with the function
417 “*fitme*” (67). We tested the effect of mutation on bacterial load within the host by comparing raw
418 data (*i.e.* the counts of bacterial colony (CFU) in a 5 μ l drop over several dilutions - only in drops
419 containing less than 50 CFU). Dilution and volume were included in the model as fixed offsets. As
420 before, we modelled fluctuations among experiments or replicate variants as random effects. An
421 additional random effect was added to take into account the count uncertainty among drops for a
422 same individual host.

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607
608

609 **Figure legends**

610 **Figure 1: Description of *lrp* mutations. The *lrp* gene is made of two domains, HTH and RAM.**

611 The study used four *lrp* mutants each with a single point mutation that strains 23, 42, 44 and 51 do
612 not carry. Strains 36 and 39 carry different nonsense mutations in codon position 53 leading to a
613 frameshift (FS). Strains 25 and 29 carry each a missense mutation in codon position 120 leading to
614 different amino acids. Strain 40 carries a nonsense mutation (duplication) in codon position 124
615 leading to a stop codon (*).

616 **Figure 2: Survival of adult *Drosophila melanogaster* upon infection with wildtype or *lrp***
617 **mutant *Xenorhabdus nematophila*.**

618 **A-** Survival over time of hosts when injected with 1000 bacteria. Wildtype bacteria, represented
619 individually by blue dotted lines, always killed their hosts faster than *lrp* mutants, represented
620 individually by red dotted lines. Solid lines represent the pooled wildtype (blue) and mutant (red)
621 strains. Black dashed lines show the LT50 of wildtype strains. Number of host individuals are
622 mentioned between brackets. **B-** Virulence of each bacterial strains. Each dot represents the death
623 hazard ratio relative to sham infection calculated from the cox model used to analyse the survival in
624 A. Blue represents infection with wildtype bacterial strains and red the mutant strains. Reference of
625 strain numbers are detailed in Figure 1.

626 **Figure 3: Bacterial success during the first step of the infection.**

627 **A-** Bacterial load estimated at 8 hours post-injection in immune (Imd) deficient flies (genotype
628 *Dredd*⁵⁵) and in the genetic background control (*w¹¹¹⁸*). Letters represent the significant differences
629 between loads of different bacterial strains in hosts with a functional immune system. Wildtype
630 bacterial strains reached a higher density than strains with a mutation in *lrp*. Only mutant strain 29
631 is intermediate. p indicates the p-value of the difference in bacterial load between immune deficient
632 hosts and healthy hosts. Unlike other strains, those with a nonsense *lrp* mutation proliferated better
633 in immune deficient host. Experiments were replicated with three (#23 and #29) or five (all other
634 strains) different overnight bacterial cultures and fly batches. **B-** Bacterial load at injection and
635 8hours post injection in immune deficient and healthy host. p indicates the p-value of the interaction
636 between time and genotype testing for the difference in proliferation. This approach validates that
637 the strain 40, with a nonsense *lrp* mutation, proliferates less in presence of immune system.
638 Experiments were replicated with three different overnight bacterial cultures and fly batches. **C-**
639 Correlation between early growth in wildtype hosts of the bacterial strains with or without mutation
640 and the hazard ratio (i.e. virulence) extracted for survival analysis in Figure 2. The virulence

641 correlated strongly with the speed of proliferation at the initiation of the infection. **D-** Correlation
642 between early growth in wildtype hosts of the bacterial strains with their early growth in immune
643 deficient host. Solid line represents a perfect correlation (i.e. when the immune system does not
644 affect the bacterial growth). Departure from the line ($y=x$) indicates a difference in proliferation
645 when the host was immunodeficient. **E-** Correlation between the effect of the immune system on
646 proliferation (i.e. estimate of the interaction effect between time and genotype) and the hazard ratio.
647 Dotted line represents the absence of difference between proliferation in immune deficient hosts
648 and in healthy host. Values are the estimates extracted from the analysis in B. In all panel, blue
649 represents wildtype strains and red represents *lrp* mutants. Coloured triangles represent the mean
650 per replicates.

651 **Figure 4: Bacterial load upon death (BLUD) estimated approximately 12h to 30h after**

652 injection in wildtype hosts.

653 **A-** Mutant strains reached the same BLUD than wildtype strains. Experiments were replicated with
654 three different overnight bacterial cultures and fly batches. **B-** Correlation between BLUD and
655 hazard ratio. Mutant and wildtype strains reached the same BLUD but wildtype strains had a higher
656 hazard ratio and reached the BLUD about 10 hours earlier, suggesting that *lrp* mutations affect
657 virulence but not pathogenicity. Coloured triangles represent the mean of the replicates.

658 **Figure 5: Proliferation during the second step of infection, within the dead host**

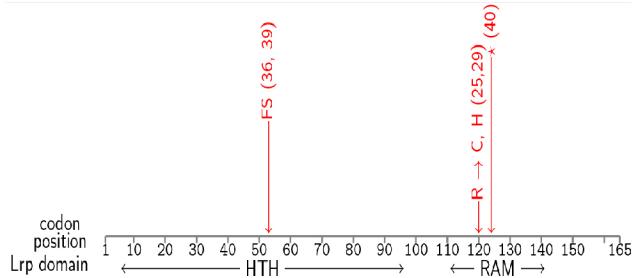
659 **A-** Proliferation between death and 24 hours later in wildtype host and **B-** model estimate of growth
660 post death (i.e. estimate of the parameter “Time” in the model to analyse A). Unlike when the host
661 was alive, *lrp* mutant strains had a higher density than wildtype strains when the host was dead.
662 Missense mutation strains (light red) did not proliferate differently in dead hosts than nonsense
663 mutation strains (dark red). Experiments were replicated with two (#23, #36, #42 and #51), three
664 (#44) or four (#25, #29, #40) different overnight bacterial cultures and fly batches.

665 **Figure 6: Trade-off between proliferation in first and second step of infection.**

666 Correlation between proliferation at the start of the infection and within the dead host. While
667 wildtype strains proliferated the best at the initiation of the infection, they were performing poorly
668 in dead host. Conversely, *lrp* mutant strains proliferated better in dead host but lost the ability to
669 proliferate fast at the initiation of the infection.

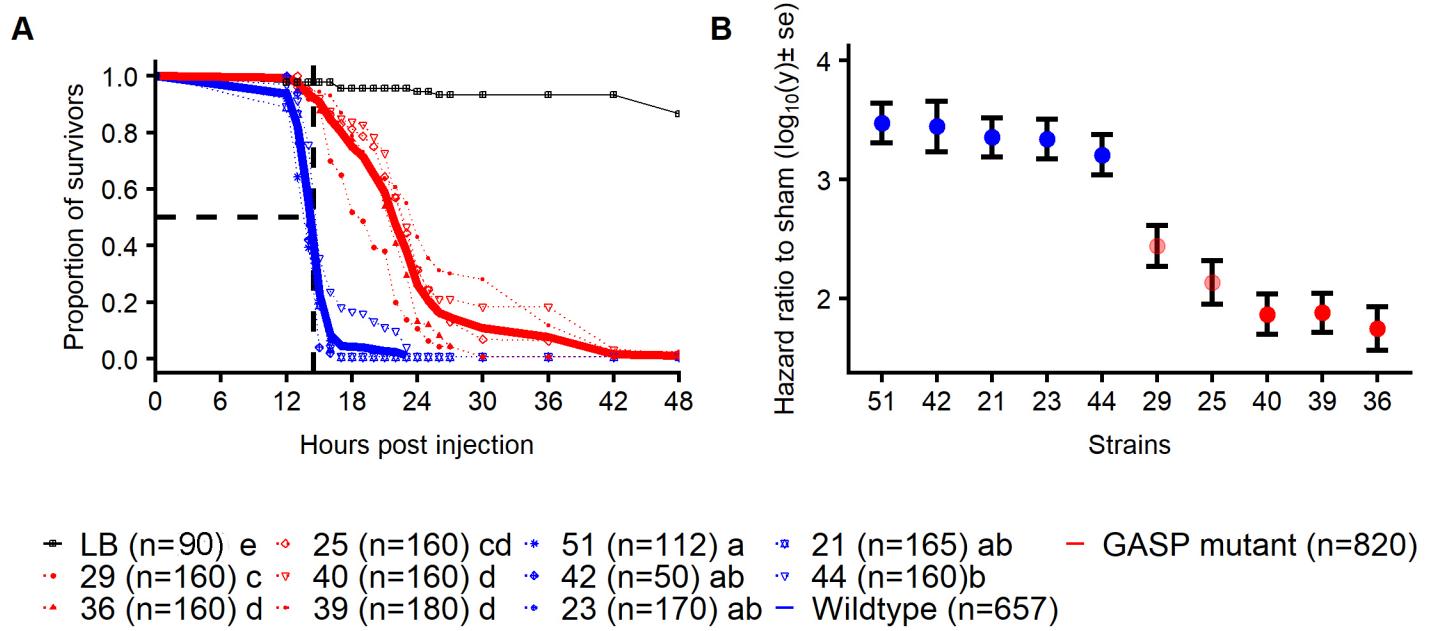
670 Correlation between proliferation at the start of the infection and within the dead host. While
671 wildtype strains proliferated the best at the initiation of the infection, they were performing poorly
672 in dead host. Conversely, *lrp* mutant strains proliferated better in dead host but lost the ability to
673 proliferate fast at the initiation of the infection.

Figure 1



*Figure 1: Description of *lrp* mutations. The *lrp* gene is made of two domains, HTH and RAM. The study used four *lrp* mutants each with a single point mutation that strains 23, 42, 44 and 51 do not carry. Strains 36 and 39 carry different nonsense mutations in codon position 53 leading to a frameshift (FS). Strains 25 and 29 carry each a missense mutation in codon position 120 leading to different amino acids. Strain 40 carries a nonsense mutation (duplication) in codon position 124 leading to a stop codon (*).*

Figure 2



*Figure 2: Survival of adult *Drosophila melanogaster* upon infection with wildtype or *Irp* mutant *Xenorhabdus nematophila*. A- Survival over time of hosts when injected with 1000 bacteria. Wildtype bacteria, represented individually by blue dotted lines, always killed their hosts faster than *Irp* mutants, represented individually by red dotted lines. Solid lines represent the pooled wildtype (blue) and mutant (red) strains. Black dashed lines show the LT50 of wildtype strains. Number of host individuals are mentioned between brackets. B- Virulence of each bacterial strains. Each dot represents the death hazard ratio relative to sham infection calculated from the cox model used to analyse the survival in A. Blue represents infection with wildtype bacterial strains and red the mutant strains. Reference of strain numbers are detailed in Figure 1.*

Figure 3

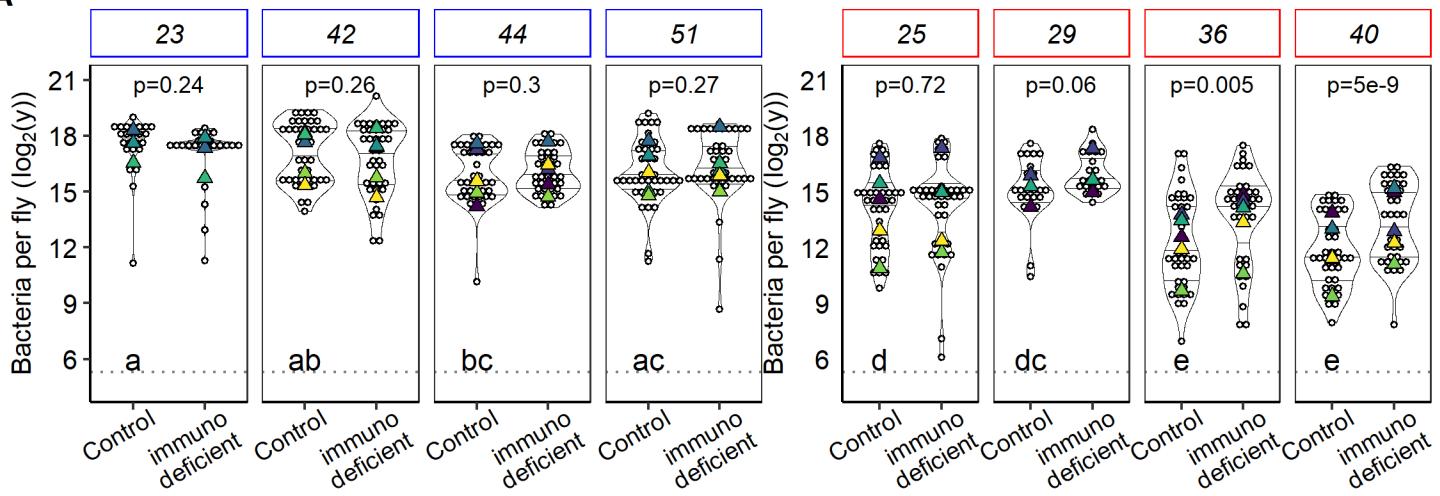
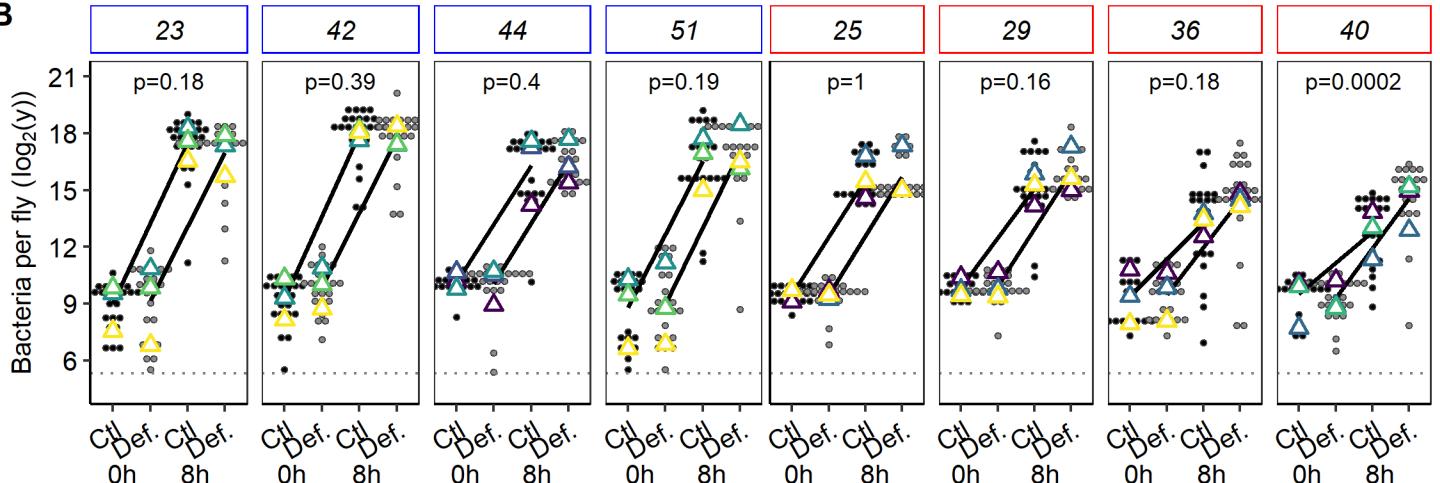
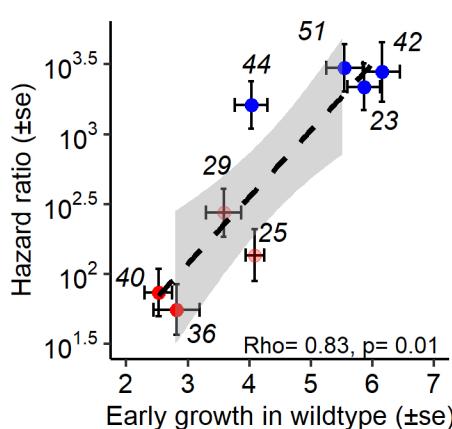
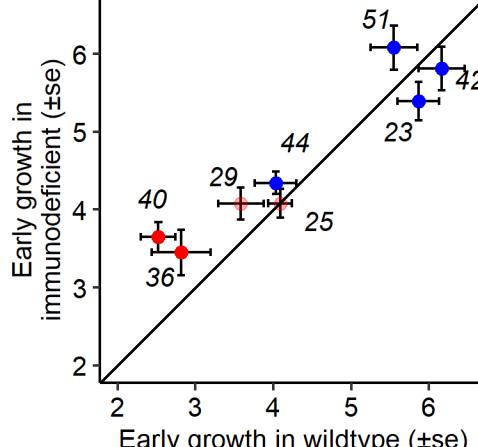
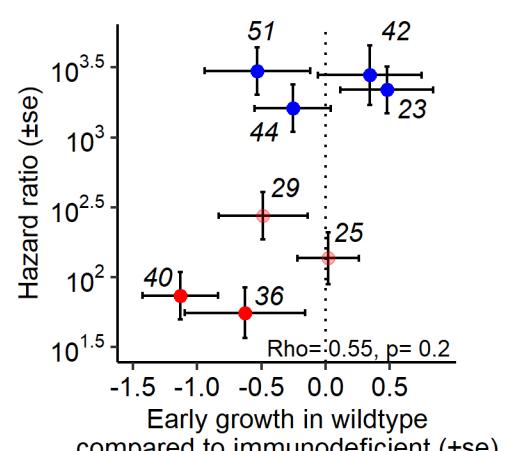
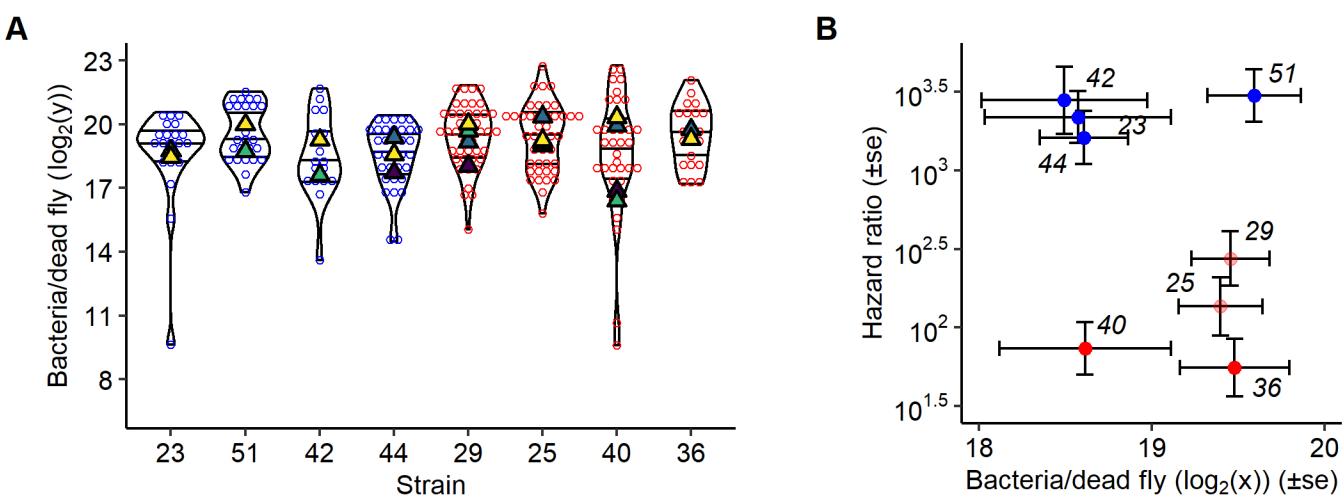
A**B****C****D****E**

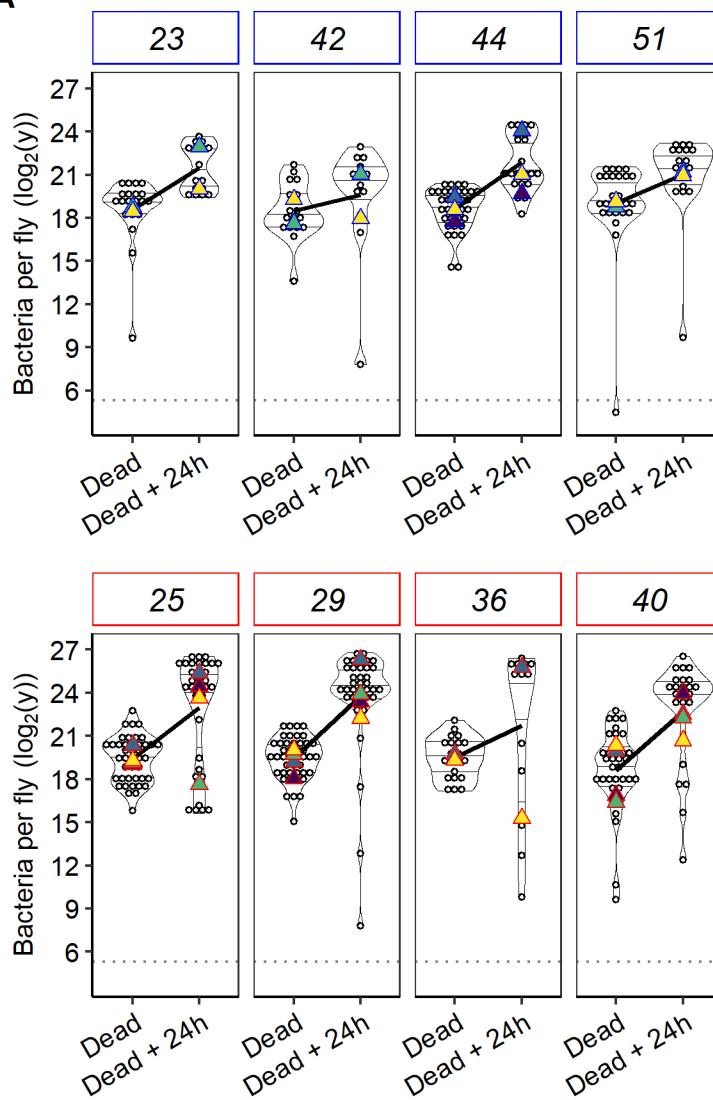
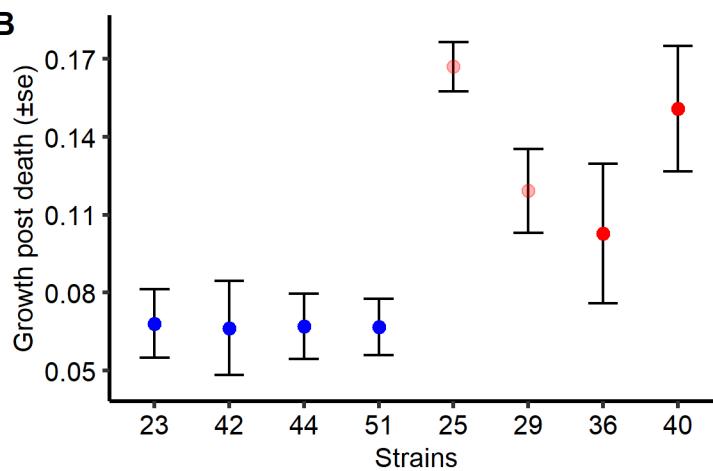
Figure 3: Bacterial success during the first step of the infection. A- Bacterial load estimated at 8 hours post-injection in immune (*Imd*) deficient flies (genotype *Dredd55*) and in the genetic background control (w1118). Letters represent the significant differences between loads of different bacterial strains in hosts with a functional immune system. Wildtype bacterial strains reached a higher density than strains with a mutation in *lrp*. Only mutant strain 29 is intermediate. p indicates the p-value of the difference in bacterial load between immune deficient hosts and healthy hosts. Unlike other strains, those with a nonsense *lrp* mutation proliferated better in immune deficient host. Experiments were replicated with three (#23 and #29) or five (all other strains) different overnight bacterial cultures and fly batches. B- Bacterial load at injection and 8hours post injection in immune deficient (Def.) and healthy host (Ctl). p indicates the p-value of the interaction between time and genotype testing for the difference in proliferation. This approach validates that the strain 40, with a nonsense *lrp* mutation, proliferates less in presence of immune system. Experiments were replicated with three different overnight bacterial cultures and fly batches. C- Correlation between early growth in wildtype hosts of the bacterial strains with or without mutation and the hazard ratio (i.e. virulence) extracted for survival analysis in Figure 2. The virulence correlated strongly with the speed of proliferation at the initiation of the infection. D- Correlation between early growth in wildtype hosts of the bacterial strains with their early growth in immunodeficient host. Solid line represents a perfect correlation (i.e. when the immune system does not affect the bacterial growth). Departure from the line ($y=x$) indicates a difference in proliferation when the host was immunodeficient. E- Correlation between the effect of the immune system on proliferation (i.e. estimate of the interaction effect between time and genotype) and the hazard ratio. Dotted line represents the absence of difference between proliferation in immune deficient hosts and in healthy host. Values are the estimates extracted from the analysis in B. In all panel, blue represents wildtype strains and red represents *lrp* mutants. Colored triangles represent the mean per replicates.

Figure 4



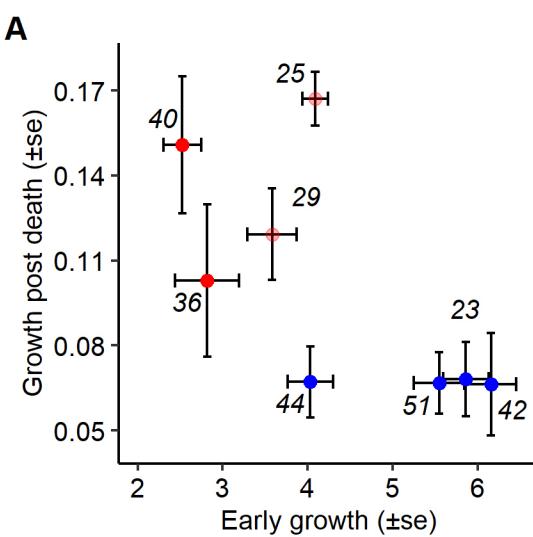
Bacterial load upon death (BLUD) estimated approximately 12h to 30h after injection in wildtype hosts. A- Mutant strains reached the same BLUD than wildtype strains. Experiments were replicated with three different overnight bacterial cultures and fly batches. B- Correlation between BLUD and hazard ratio. Mutant and wildtype strains reached the same BLUD but wildtype strains had a higher hazard ratio and reached the BLUD about 10 hours earlier, suggesting that *lrp* mutations affect virulence but not pathogenicity. Colored triangles represent the mean of the replicates.

Figure 5

A**B**

*Proliferation during the second step of infection, within the dead host. A- Proliferation between death and 24 hours later in wildtype host and B- model estimate of growth post death (i.e. estimate of the parameter 'Time' in the model to analyse A). Unlike when the host was alive, *Irp* mutant strains had a higher density than wildtype strains when the host was dead. Missense mutation strains (light red) did not proliferate differently in dead hosts than nonsense mutation strains (dark red). Experiments were replicated with two (#23, #36, #42 and #51), three (#44) or four (#25, #29, #40) different overnight bacterial cultures and fly batches. Colored triangles represent the mean of the replicates.*

Figure 6



*Figure 6: Trade-off between proliferation in first and second step of infection. Correlation between proliferation at the start of the infection and within the dead host. While wildtype strains proliferated the best at the initiation of the infection, they were performing poorly in dead host. Conversely, *lrp* mutant strains proliferated better in dead host but lost the ability to proliferate fast at the initiation of the infection.*