Bacteria Are a Major Determinant of Orsay Virus Transmission and Infection in *Caenorhabditis elegans*

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Abstract

The microbiota is a key determinant of the physiology and antiviral immunity of animal hosts. The factors governing the transmissibility of viruses between susceptible hosts are incompletely understood. Bacteria serve as food for *Caenorhabditis elegans* and represent an integral part of the natural environment of *C. elegans.* We determined the effects of bacteria isolated with *C. elegans* from its natural environment on the transmission of Orsay virus in *C. elegans* using quantitative virus transmission and host susceptibility assays. We observed that *Ochrobactrum* species promoted Orsay virus transmission, whereas *Pseudomonas lurida* MYb11 attenuated virus transmission relative to the standard laboratory bacterial food *Escherichia coli* OP50. We found that pathogenic *Pseudomonas aeruginosa* strains PA01 and PA14 further attenuated virus transmission. We determined that the amount of Orsay virus required to infect 50% of a *C. elegans* population on *P. lurida* MYb11 compared with *Ochrobactrum vermis* MYb71 was dramatically increased, over three orders of magnitude. Host susceptibility was attenuated even further in presence of *P. aeruginosa* PA14. Genetic analysis of the determinants of *P. aeruginosa* required for attenuation of *C. elegans* susceptibility to Orsay virus infection revealed a role for regulators of quorum sensing. Our data suggest that distinct constituents of the *C. elegans* microbiota and potential pathogens can have widely divergent effects on Orsay virus transmission, such that associated bacteria can effectively determine host susceptibility versus resistance to viral infection. Our study provides quantitative evidence for a critical role for tripartite host-virus-bacteria interactions in determining the transmissibility of viruses among susceptible hosts.

37 **Introduction**

38 Viruses are ubiquitous and abundant[1,2]. Infection can have profound consequences for
39 the health of an individual host. The ability of viruses to transmit from one individual to another
40 can scale these consequences causing morbidity and mortality throughout whole populations.
41 Many factors influence virus transmission[3–5]. Abiotic factors such as temperature[6] and humidity[7]
42 and biotic factors such as viral load[8,9] and host immune status[10,11] all interact to determine
43 transmission rates. Despite these findings, the determinants of virus transmissibility remain
44 incompletely understood.

45 The microbiota has emerged as a host-associated factor that modulates multiple aspects
46 of virus infection and thereby alters transmission rates among host organisms[12–14]. In general,
47 the microbiota is critical for the proper development of the immune system and for the effective
48 activation of antimicrobial immune responses even at sites distal to microbiota colonization[15].
49 More specifically, bacteria and bacterial surface structures such as lipopolysaccharide and
50 peptidoglycan have been shown to stabilize poliovirus and reovirus *in vitro*[13,16]. These
51 observations likely explain why microbiota depletion by antibiotic treatment was sufficient to
52 provide protection against the same viruses in mice[13]. The bacterial symbiont *Wolbachia* protects
53 numerous insect species from multiple viruses either by upregulating antiviral defenses or
54 competing for intracellular nutrients[17–21]. Individual bacteria have also been found to enhance
55 viral infection; *Serratia marcescens* promoted infection of the mosquito *Aedes aegypti* by
56 Dengue, Zika, and Sindbis viruses by secreting a protein, enhancin, that degrades the mucus layer
57 covering epithelial cells[14].

58 *Caenorhabditis elegans* is a nematode often found in microbially rich environments such
59 as rotting vegetation[22]. The first naturally occurring virus capable of infecting the *C. elegans* was
60 isolated from Orsay, France[23]. Orsay virus is a part of a group of nematode infecting viruses
61 closely related to the *Nodaviridae* family of viruses which infect arthropods and fish[23,24]. Like
62 other Nodaviruses, Orsay virus is a positive-sense, single-stranded RNA virus with a bipartite
63 genome[23]. Transmission of Orsay virus occurs horizontally through the fecal-oral route.
64 Fluorescence *in situ* hybridization and immunofluorescence imaging has revealed that Orsay virus
65 solely infects *C. elegans* intestinal cells[23,25]. Viral infection activates host defense mechanisms
66 including the RNA-interference response and a transcriptional program known as the
67 intracellular pathogen response[23,26–29].

68 *C. elegans* is a bacterivore and is propagated in the laboratory on lawns of *Escherichia coli*
69 OP50. *Pseudomonas aeruginosa,* an opportunistic pathogen of humans, is found in the soil and
70 water and can also infect *C. elegans*[30–32]. Infection of *C. elegans* with *P. aeruginosa* activates
71 innate immunity and stress responses, as well as behavioral avoidance responses[33–35]. Recently,
72 the bacteria resident in the natural environment of *C. elegans* in the wild have been of increasing
73 interest[22,36–38]. The intestinal lumen of free-dwelling *C. elegans* is occupied by taxonomically and
74 functionally diverse bacteria that can affect *C. elegans* fitness and physiology[36,37,39].

75 In this study, we sought to understand how bacteria that are constituents of the *C.*
76 *elegans* microbiota quantitatively affect the transmission of Orsay virus in *C. elegans.* We
77 observed that monoaxenic cultures of different bacterial species had widely divergent effects on
78 the transmission of Orsay virus and conducted genetic analysis of the bacterial determinants

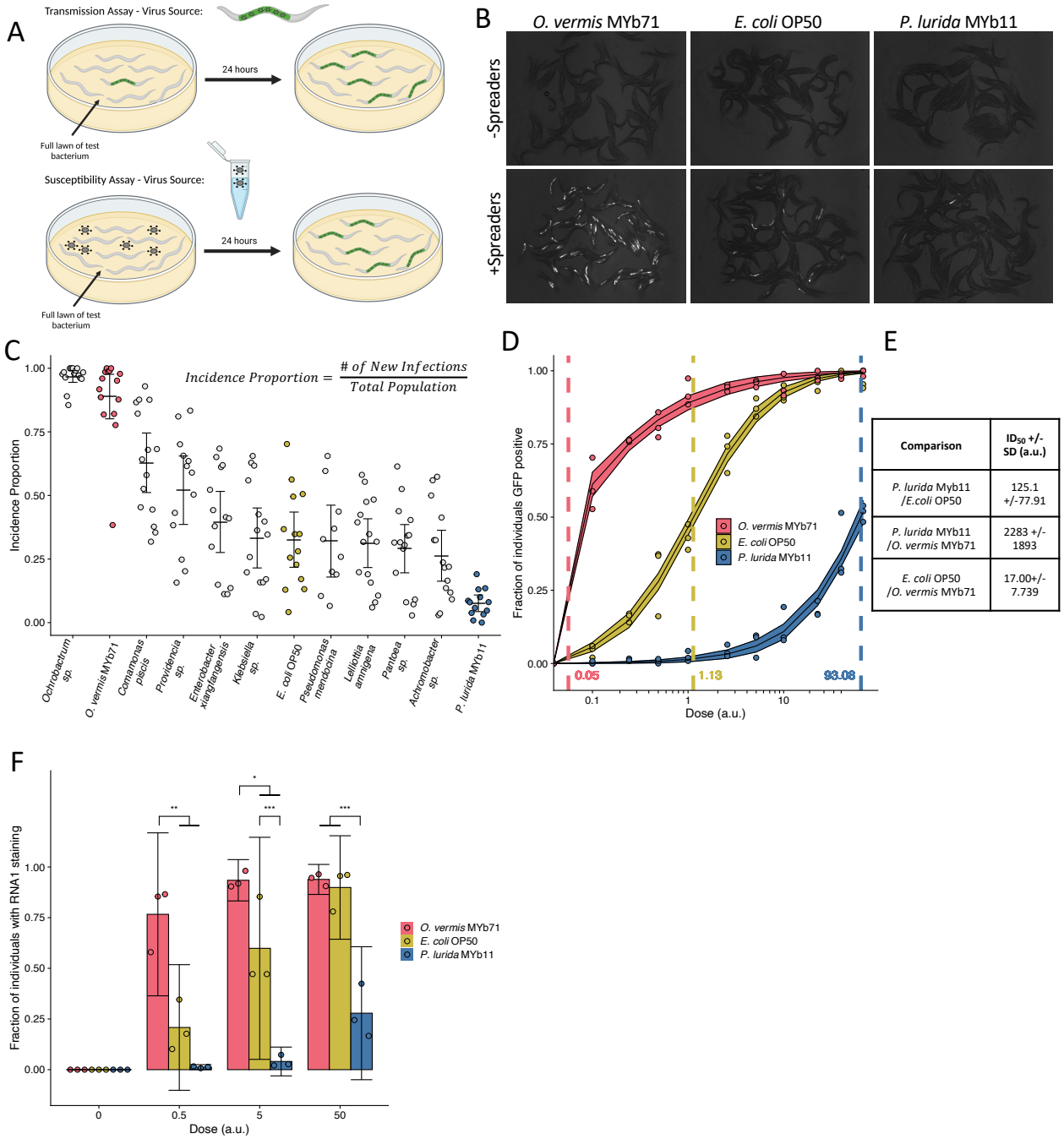
79 involved in modulating virus transmission. Our data point to a key species-specific role for
80 bacteria as critical determinant of virus transmission.
81
82 **Results**
83 **Wide variation in the effects of bacteria on the transmission of Orsay virus.**
84 Orsay virus spreads horizontally through the fecal-oral route and transmission can be
85 observed spreading from a single animal to a population of animals on a plate[40]. We sought to
86 assess the impact of bacteria on Orsay virus transmission rates and utilized a collection of
87 bacteria isolated from the environment with wild *C. elegans* to assemble a panel of Gram-
88 negative bacteria for comparison with the standard laboratory bacterial food, *Escherichia coli*
89 OP50[36,37,39,41]. We set up a transmission assay by placing infected animals ("spreaders") together
90 with uninfected animals on a monoaxenic lawn of each bacteria and examining the incidence
91 proportion, or, the number of new infections produced after 24 h as determined by detection of
92 induction of the *pals-5p::GFP* reporter, which is induced by infection with Orsay virus (Fig. 1A-
93 C)[27]. We observed a wide range in the measured incidence proportion (Fig. 1B and 1C). Exposure
94 to many of the naturally-associated bacterial strains resulted in transmission that was
95 comparable to the incidence proportion observed with *E. coli* OP50 with some prominent
96 exceptions (Fig. 1B and 1C). Two *Ochrobactrum* species promoted virus infection in nearly all
97 individuals in the transmission assay and increased the incidence proportion 2.7-fold and 2.9-fold
98 compared to *E. coli* OP50 (Fig. 1B and 1C). We confirmed that transmission from the initial
99 spreader animals in the assay, and not multiple rounds of infection, was responsible for the
100 increased incidence proportion observed in the presence of *Ochrobactrum vermis* MYb71 (Supp.
101 Fig. 1A and 1B). On the other hand, the presence of *P. lurida* MYb11 reduced the incidence

102 proportion 4.1-fold compared to *E. coli* OP50 and 11-fold compared to *O. vermis* MYb71 (Fig. 1B
103 and C). These results present a striking divergence in the effects of individual bacterial
104 constituents of the *C. elegans* microbiota on the level of transmission of Orsay virus from infected
105 animals.

106 Of further note, we observed a substantial amount of variability in the incidence
107 proportion in the presence of most bacteria (Fig. 1C). Considering both the spreaders and
108 uninfected animals are isogenic populations, the variability we observe between technical and
109 experimental replicates may be due to the inherently stochastic elements of virus transmission.
110 It is striking that the two *Ochrobactrum* species and *P. lurida* MYb11 were exceptions to this rule,
111 as it suggests these bacteria somehow mitigate the semi-random elements of virus transmission
112 under these conditions.

113 We reasoned that increased transmission of Orsay virus could be due to bacterial effects
114 on the shedding of virus by infected spreader animals or bacterial modulation of host
115 susceptibility to viral infection. To determine quantitatively how different bacterial species
116 modulate host susceptibility to viral infection independently of the potential differential effects
117 of bacteria on host shedding of viruses, we added a fixed amount of Orsay virus to plates of each
118 bacterial species and monitored infection using the *pals-5p::GFP* reporter. Specifically, we added
119 Orsay virus in doses that ranged across four orders of magnitude and then quantified the fraction
120 of individuals that became infected after 24 h (Fig. 1A and 1D). At lower doses of Orsay virus, the
121 presence of *O. vermis* MYb71 resulted in a higher fraction of infected animals than those
122 observed in the presence of *E. coli* OP50 or *P. lurida* MYb11 (Fig. 1D). At higher doses of Orsay
123 virus, the presence of *P. lurida* MYb11 resulted in a reduced fraction of infected animals

124 compared to *O. vermis* MYb71 and *E. coli* OP50 (Fig. 1D). We further quantified these impacts on
125 host susceptibility by calculating the dose of Orsay virus required to infect 50% of the population
126 after 24 h of exposure, which we defined as the ID_{50} (Fig. 1D and 1E). On average we observed
127 that the ID_{50} in the presence of *E. coli OP50* was 17-fold higher than the ID_{50} in the presence of
128 *O. vermis* MYb71 (Fig. 1E). The ID_{50} observed in the presence of *P. lurida* was 120-fold and 2300-
129 fold higher than the ID_{50} in the presence of *E. coli* OP50 or *O. vermis* MYb71 respectively (Fig. 1E).
130 We corroborated our observations from the *pals-5p::GFP* reporter by scoring a
131 susceptibility assay using fluorescence *in situ* hybridization to detect the RNA1 segment of the
132 Orsay virus genome in the intestinal cells of infected animals (Fig. 1F). As expected, we observed
133 that at lower doses the presence of *O. vermis* MYb71 resulted in a greater fraction of infected
134 animals compared to *E. coli* OP50 or *P. lurida* MYb11, while at higher doses the presence of *P.*
135 *lurida* MYb11 resulted in a reduced fraction of infected animals (Fig. 1F). Together these data
136 establish that individual members of the *C. elegans* microbiota can modulate host susceptibility
137 to Orsay virus over three orders of magnitude with dramatic consequences for the
138 transmissibility of Orsay virus.



Comparison	ID_{50} +/- SD (a.u.)
P. lurida Myb11 /*E.coli* OP50	125.1 +/-77.91
P. lurida MYb11 /*O. vermis* MYb71	2283 +/- 1893
E. coli OP50 /*O. vermis* MYb71	17.00+/- 7.739

139

Figure 1: *Ochrobactrum* species and *P. lurida* MYb11 divergently modulate Orsay virus transmission and infection rates.
(A) Schematic representation of the transmission and susceptibility assays: Transmission can be assessed by combining infected spreader individuals (green nematode), uninfected reporter individuals (white nematodes), and various bacteria. Susceptibility can be assessed by combining uninfected reporter individuals with exogenous Orsay virus, and various bacteria. 24 h later, infection is assessed. This schematic was made using Biorender. (B) Representative images of GFP expression among individuals exposed to spreaders or no spreaders in the presence of *O. vermis* MYb71, *E. coli* OP50, or *P. lurida* MYb11. (C) Incidence proportion, calculated as indicated, of Orsay virus transmission quantified on different bacteria from the environment of *C. elegans*. Data shown are from three experiments combined, each dot represents the incidence proportion from a single plate. (D) Dose response curves of *C. elegans* to exogenous Orsay virus. The dashed line indicates the calculated dose at which 50% of the population was infected, the ID_{50}, the exact value which is given above the x-axis for each bacterium. The solid curves represent the 95% confidence interval of the modeled log-logistic function while in the presence of each bacterium. Data are from a single representative experiment, Dots represent individual plates. ((a.u.), arbitrary units). (E) Table shows the mean +/- the standard deviation of the ID_{50} ratio of the indicated bacteria measured in three experiments as in (D). (F) The fraction of individuals with staining as assessed by Fluorescence *in situ* hybridization targeting the RNA1 segment of Orsay virus. Data shown are from three experiments combined, each dot represents three pooled technical replicates from a susceptibility assay as in (D). For all plots the black bar is the mean and error bars are the 95% confidence interval (C.I.). p-values determined using one-way ANOVA followed by Tukey's Honest Significant Difference (HSD) test (NS non-significant, *p<0.05, **p<0.01, ***p<0.001).

140 ***P. aeruginosa* attenuates Orsay virus transmission.**

141 In view of the effect of *P. lurida* MYb11 on attenuating Orsay virus infection of *C. elegans,*
142 we examined the effect of the distantly related bacterium, *Pseudomonas aeruginosa,* an
143 opportunistic pathogen of humans that has been characterized extensively[30,42]. We observed
144 that in the presence of either *P. aeruginosa* strains PA01 or PA14, transmission from spreader
145 animals to uninfected individuals was nearly completely blocked and the incidence proportion
146 was 11-fold and 32-fold lower than that observed in the presence of *E. coli* OP50, respectively
147 (Fig. 2A). The attenuating effect of *P. aeruginosa* PA01 and PA14 on virus transmission was
148 further reduced 3.1-fold and 9.5-fold, respectively, compared to the incidence proportion
149 observed in the presence of *P. lurida* MYb11 (Fig. 2A). Once more we observed substantial
150 variation in the incidence proportion in the presence of *E. coli* OP50, while the incidence
151 proportions in the presence of either *O. vermis* MYb71, *P. aeruginosa* PA01, or *P. aeruginosa*
152 PA14 had minimal variation, suggesting a potent overall effect on transmission under these
153 conditions (Fig 2A).

154 We confirmed that host susceptibility to Orsay virus was reduced in the presence of *P.*
155 *aeruginosa* by performing susceptibility assays at two doses of exogenous Orsay virus. At each
156 dose, fewer animals were infected following exposure to equivalent doses of Orsay virus in the
157 presence of *P. aeruginosa* PA01, PA14, or *P. lurida* MYb11 as compared to the fraction of animals
158 infected in the presence of *E. coli* OP50 (Fig. 2B). At the highest dose of virus used, we still
159 observed robust attenuation of infection in the presence of *P. aeruginosa* PA14 as 0.5% of the
160 animals were infected as compared to 54%, 70%, and 97% in the presence of *P. aeruginosa* PA01,
161 *P. lurida* MYb11, or *E. coli* OP50, respectively (Fig. 2B). We confirmed that no individuals were

162 detectably infected with Orsay virus while in the presence of *P. aeruginosa* PA14 using FISH
163 staining (Fig. 2C). FISH additionally confirmed that the presence of *P. aeruginosa* PA01 and *P.*
164 *lurida* MYb11 attenuated average infection to 33% and 62% of the population respectively
165 compared to *E. coli* OP50 which supported infection of 85% of the population. Further, *P.*
166 *aeruginosa* PA01, *P. lurida* MYb11, and *E. coli* OP50 all supported higher levels of infection than
167 *P. aeruginosa* PA14. (Fig. 2C). Together these results demonstrate a striking capacity of the
168 presence of *P. lurida and P. aeruginosa* species to sharply reduce and even effectively block, host
169 susceptibility to infection with Orsay virus.

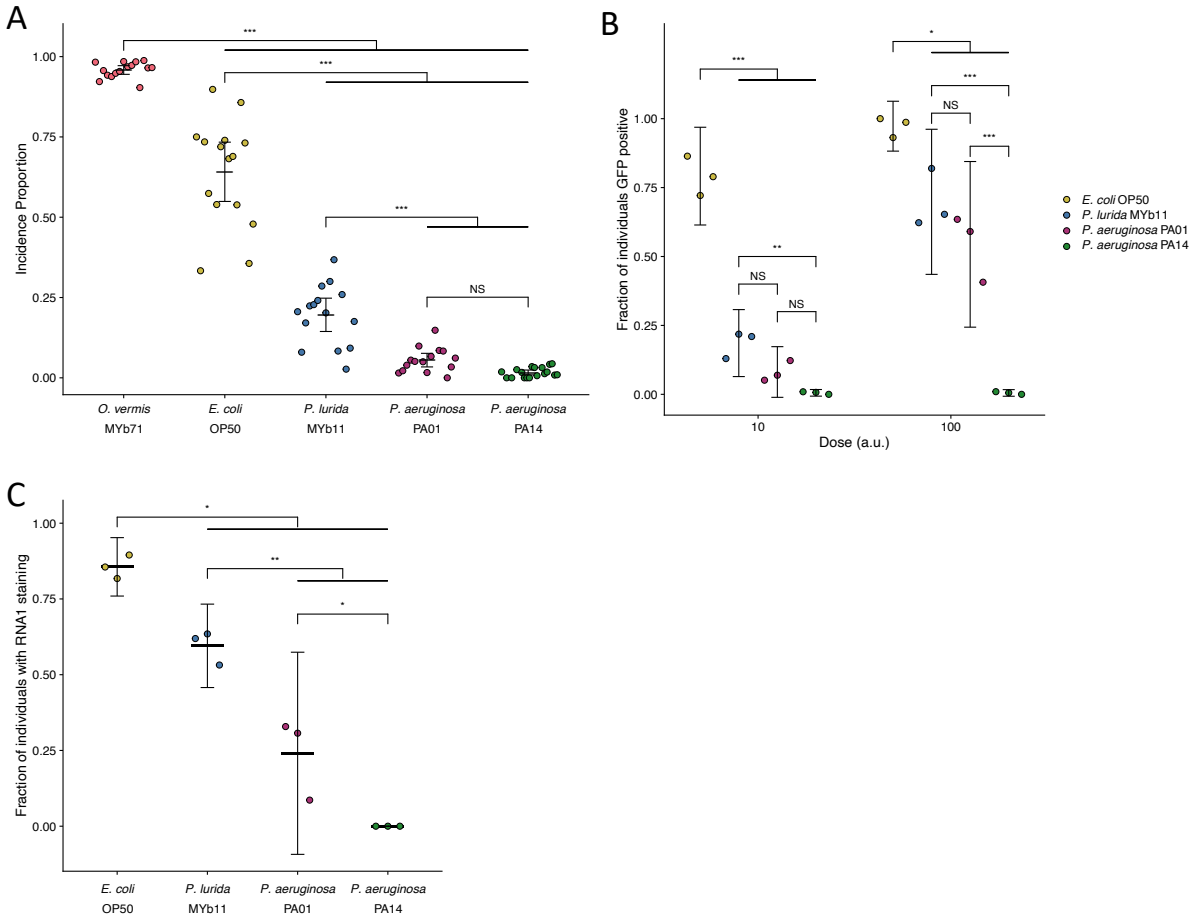


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Figure 2: *P. aeruginosa* attenuates Orsay virus transmission and infection rates.

171 (A) Incidence proportion of Orsay virus transmission quantified on different bacteria from *C. elegans*' environment and *P. aeruginosa* PA01 and *P. aeruginosa* PA14. Data shown are from three experiments combined, each dot represents the incidence proportion from a single plate. (B) The fraction of individuals GFP positive following exposure to two doses of exogenous Orsay virus. Data are from a single representative experiment and dots represent individual plates. (C) The fraction of individuals with staining following exposure to 100a.u. Orsay Virus as assessed by fluorescence *in situ* hybridization targeting the RNA1 segment of the Orsay Virus genome. Data shown are from three experiments combined, each dot represents three pooled technical replicates from an experiment. For all plots the black bar is the mean and error bars are the 95% confidence interval (C.I.). p-values determined using one-way ANOVA followed by Tukey's Honest Significant Difference (HSD) test (NS non-significant, *p<0.05, **p<0.01, ***p<0.001).

172 **Attenuation of Orsay virus transmission by *P. lurida* and *P. aeruginosa* is not due to effects on**
173 **Orsay virus replication.**

174 We considered the possibility that the attenuation of Orsay virus transmission in the
175 presence of *P. lurida* and *P. aeruginosa* strains might be due to inhibitory effects of the bacteria
176 on the replication of Orsay virus once transmitted to a susceptible animal host. To evaluate this
177 possibility, we made use of a plasmid-based system in which viral RNA1 is expressed through a
178 transgene introduced into *C. elegans,* so that replication of RNA1 can be assessed independent
179 of the entry of exogenous virus into the host[43]. In this system, the Orsay virus RNA1 segment,
180 which encodes the RNA-dependent RNA polymerase (RdRP), is expressed following heat-shock.
181 The expressed RNA1 may then be translated to produce the RdRP which can then replicate RNA1
182 through a negative-strand intermediate. The expression of RNA1 via heat-shock bypasses any
183 differences in viral entry or pre-replication steps, allowing for a direct test of RNA1 replication
184 efficiency under different conditions[43]. A plasmid expressing a mutated RdRP (RNA1[D601A])
185 incapable of supporting further RNA1 replication after the initial heat-shock serves as a control
186 for heat shock efficiency[43]. Using this system, we observed that Orsay virus RNA1 replication
187 efficiency was unaffected by the presence of *Pseudomonas* species relative to RNA1 replication
188 observed in the presence of *E. coli* OP50 (Fig. 3A). Additionally, there were no differences
189 observed in heat-shock efficiency between the different bacteria (Fig. 3A). These data suggest
190 that bacteria-induced differences in RNA1 replication in the host do not explain the substantial
191 attenuation of Orsay virus transmission and infection rates caused by *P. aeruginosa* PA01 or PA14
192 and *P. lurida* MYb11.

193 We sought to confirm the successful replication of the plasmid expressed RNA1 of Orsay
194 virus by assessing whether we could detect the replication of Orsay virus RNA1 following rare

events of natural infection of *C. elegans* in the presence of *P. aeruginosa* PA14. We exposed young adult wild-type (N2) or RNAi defective animals (*rde-1(ne219)*) to exogenous Orsay virus while in the presence of *P. aeruginosa* PA14 and quantified the amount of virus present at 2 h and 24 h post-exposure, with the difference in RNA levels at these points reflecting viral genome replication. In the N2 background viral RNA1 levels increased by 3.5-fold at 24 h relative to levels at 2 h post-exposure (Fig. 3B). Orsay virus is more effective at replicating in the *rde-1* background compared to N2 and in the *rde-1* background viral RNA1 levels increased by 9000-fold at 24 h relative to levels at 2 h post-exposure (Fig. 3B)[23]. Together, these results suggest that Orsay virus RNA1 can also replicate once natural infection is achieved in both the wild-type and *rde-1*-defective background while animals are in the presence of *P. aeruginosa* PA14. These data confirm our findings that bacteria induced differences in viral replication do not explain the substantial attenuation of Orsay virus transmission in the presence of *P. aeruginosa* PA14.

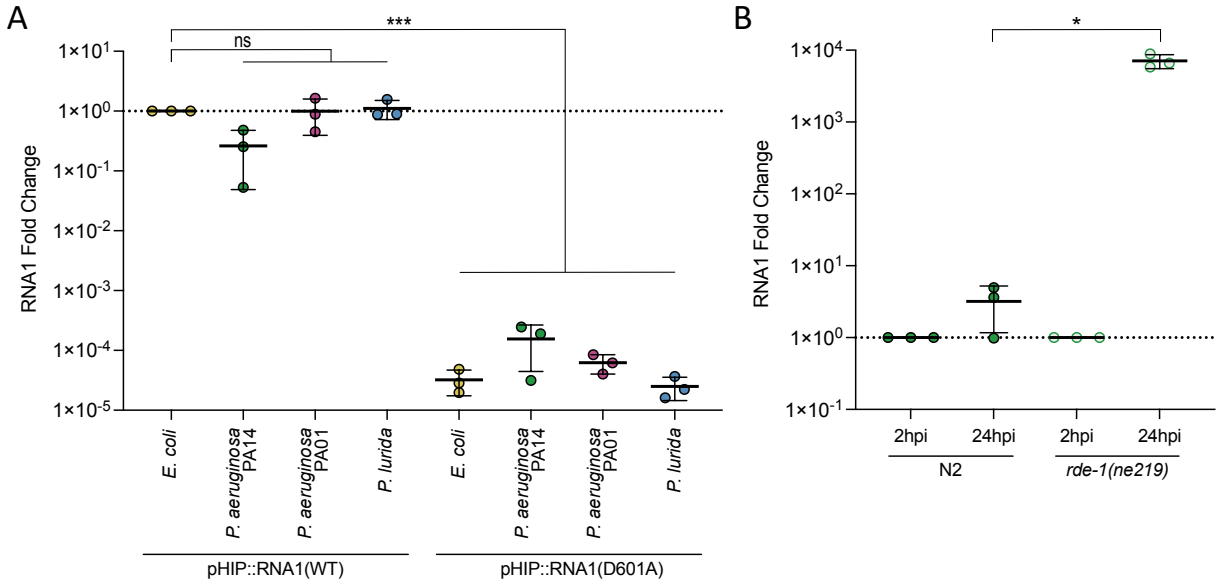

Figure 3: *P. aeruginosa* and *P. lurida* MYb11 do not eliminate Orsay virus replication.
(A) Animals carrying either a wild-type (RNA1(WT)) or replication defective (RNA1(D601A)) transgenic viral RNA replicon system were used to assess Orsay virus replication efficiency independently of virus entry. P-values determined using one-way ANOVA followed by Dunnett's test (B) Orsay virus RNA1 levels of N2 and *rde-1(ne219)* animals exposed to *P. aeruginosa* PA14 and exogenous Orsay virus 2- and 24- h post infection (hpi). P-values were determined using Welch's t-Test For each plot, each dot represents five pooled technical replicates, the black bar is the mean, error bars are the standard deviation, RNA1 levels were quantified by qPCR, and the data shown are for three independent experiments. (NS non-significant, *p<0.05, **p<0.01, ***p<0.001).

209 **Attenuation of Orsay virus transmission by *Pseudomonas* species is dependent on regulators**
210 **of bacterial quorum sensing.**

211 Extensive studies on *Pseudomonas* species have demonstrated the importance of quorum
212 sensing for regulating many community-level behavior in response to growing population
213 density[44]. *P. aeruginosa* relies upon three quorum sensing systems: *las*, *rhl*, and *pqs*. These
214 systems are arranged hierarchically, however crosstalk between them is extensive (Fig. 4A)[44]. In
215 *P. aeruginosa*, an additional layer of regulation stems from two-component regulatory systems
216 such as *gacA/gacS* which regulates numerous genes that together influence quorum sensing,
217 virulence, and biofilm development (Fig. 4A)[45–47]. *P. aeruginosa* possesses three
218 exopolysaccharide biosynthetic clusters that each can contribute to biofilm formation: *pel*, *psl*,
219 and *alg*. However, *P. aeruginosa* PA01 preferentially produces Psl while *P. aeruginosa* PA14 is
220 unable to synthesis Psl and produces Pel[48–52]. We hypothesized that quorum sensing might
221 mediate the effect of *Pseudomonas* strains to attenuate virus transmission, while differences in
222 exopolysaccharide production might mediate the enhanced attenuation of virus transmission
223 observed in the presence of *P. aeruginosa* PA14 compared with what was observed in the
224 presence of *P. aeruginosa* PA01. Therefore, we tested a panel of *P. aeruginosa* PA01 and *P.*
225 *aeruginosa* PA14 quorum sensing and biofilm mutants to determine whether quorum sensing or
226 biofilm formation was involved in the attenuation of Orsay virus infection mediated by *P.*
227 *aeruginosa.*

228 Mutations of any the regulators of the *las*, *rhl*, or *pqs* quorum sensing systems suppressed
229 the attenuation of Orsay virus infection caused by the presence of wild-type *P. aeruginosa* PA01
230 (Fig. 4B). Knockout of *gacA* in *P. aeruginosa* PA01 also suppressed the attenuation of Orsay virus
231 infection (Fig. 4B). On the other hand, mutation of any of the three exopolysaccharide production

232 pathways had no effect on infection (Fig. 4B). These data support a role for quorum sensing
233 regulated processes in reducing Orsay virus infection rates but do not implicate a role for
234 exopolysaccharide biosynthesis.

235 For *P. aeruginosa* PA14, mutation of *gacA* or *rhlR* suppressed the attenuation of Orsay
236 Virus infection observed in the presence of wild-type *P. aeruginosa* PA14 (Fig. 4C). Loss of the
237 *rhlR* regulators *rhlI* or *pqsE* alone had no effect on the attenuation of Orsay virus infection, but
238 simultaneous mutation of both *rhlI* and *pqsE* did suppress the attenuation of Orsay virus infection
239 observed in the presence of wild-type *P. aeruginosa* PA14, similar to that observed for the *rhlR*
240 mutant, suggesting *rhlI* and *pqsE* function redundantly to regulate *rhlR* in this context (Fig. 4C)[53].
241 Mutation of *lasI* or *lasR* suppressed the attenuation of Orsay virus infection to a lesser extent
242 (Fig. 4C). Independent mutation of two genes responsible for *pel* or *alg* exopolysaccharide
243 production had no effect on infection rates (Fig. 4C). These data suggest a role for quorum
244 sensing in mediating *P. aeruginosa* PA14 suppression of Orsay virus infection, as we observed for
245 *P. aeruginosa* PA01. However, our results obtained in the presence of *P. aeruginosa* PA14 suggest
246 that there may be some differential regulation of the bacterial effectors responsible in
247 comparison to *P. aeruginosa* PA01, or additional non-quorum sensing related factors that also
248 mediate suppression.

249 As *P. aeruginosa* mutants could suppress the effects of wild-type *P. aeruginosa* on *C.*
250 *elegans* infection in the presence of exogenous virus, we next confirmed that these *P. aeruginosa*
251 mutants similarly affected not just host susceptibility, but also transmission from infected
252 spreader animals. All *P. aeruginosa* PA01 quorum sensing mutants suppressed the attenuation
253 of transmission by increasing the incidence proportion >6-fold compared to the wild-type *P.*

254 *aeruginosa* PA01 (Fig. 4D). *P. aeruginosa* PA14 *rhlR* and *gacA* mutants suppressed the attenuation
255 of transmission by increasing the incidence proportion 19-fold and 17-fold respectively compared
256 to wild-type *P. aeruginosa* PA14, but a *lasI* mutant had minimal effect consistent with the pattern
257 we observed in the susceptibility assay using exogenous Orsay virus (Fig. 4D). Likewise, the
258 magnitude of suppression of the attenuation of Orsay virus transmission observed in the *P.*
259 *aeruginosa rhlR* or *gacA* mutants was greater in the *P. aeruginosa* PA01 background compared
260 to the *P. aeruginosa* PA14 background, potentially suggesting the existence of additional strain
261 specific factors that act specifically in *P. aeruginosa* PA14 to attenuate Orsay virus transmission.
262 It is additionally interesting to note that similar to the incidence proportion in the
263 presence of *E. coli* OP50, the variation in the incidence proportion in the presence of all *P.*
264 *aeruginosa* mutants increased (Fig 4D). This was true even for the incidence proportion observed
265 in the presence of *P. aeruginosa* PA14 *lasI* mutant, which while not statistically different from
266 wild-type *P. aeruginosa* PA14, had a qualitatively different distribution nonetheless (Fig 4D).

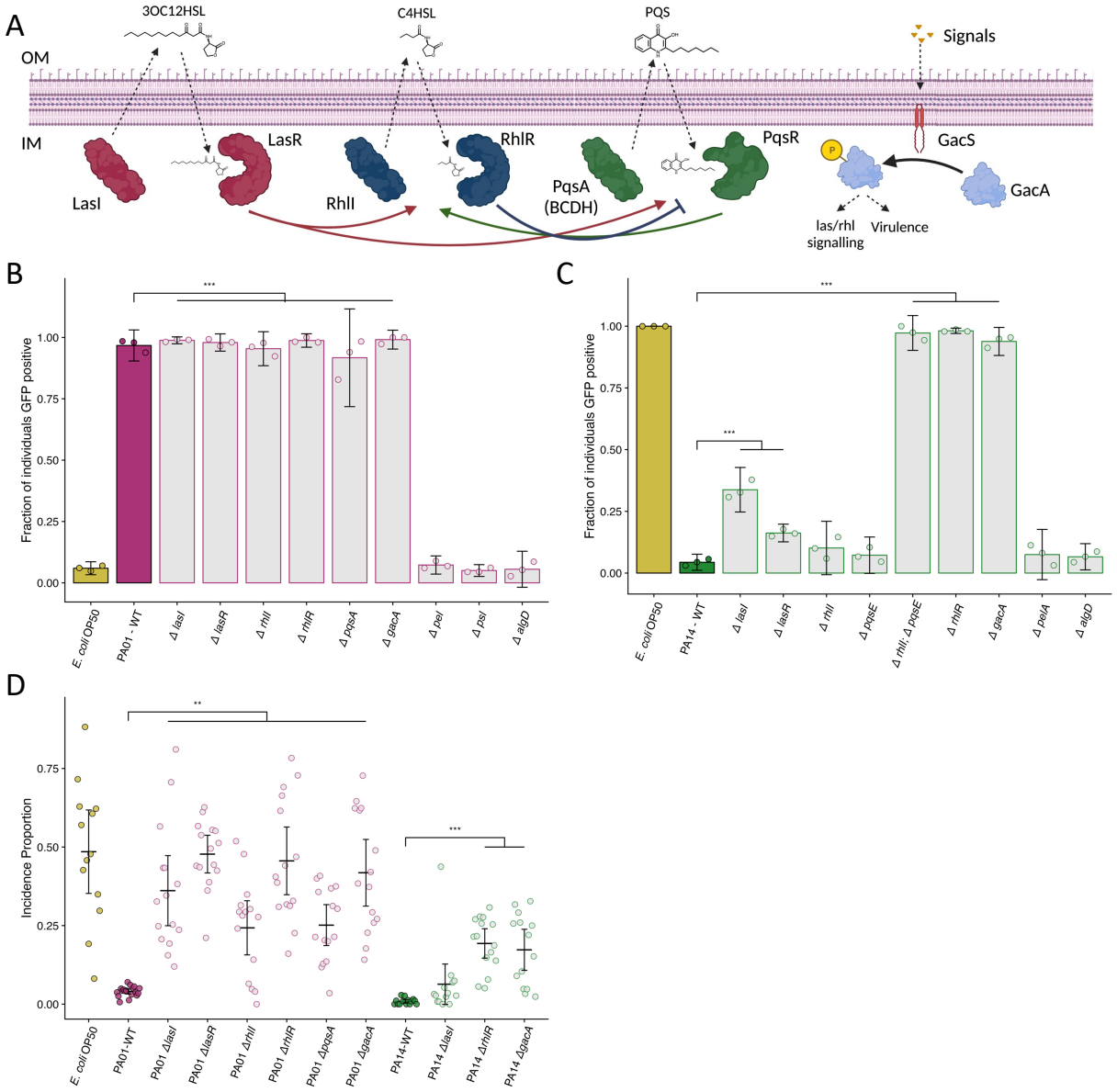

Figure 4: Mutation of *P. aeruginosa* quorum sensing regulators and the two-component response regulator *gacA* suppresses the attenuation Orsay virus infection.

(A) Diagram demonstrating the three quorum sensing systems in *P. aeruginosa*. Each system encodes the enzyme(s) (LasI, RhlI, PqsA(BCDH) to produce an autoinducer (3OC12HSL – 3-oxo-C12-homoserine lactone, C4HSL –butanoyl homoserine lactone , PQS – Pseudomonas quinolone signal) that is recognized by its cognate receptor (LasR, RhlR, PqsR) that influences gene transcription and the activity of the other quorum sensing systems. An additional level of regulation stems from the two-component *gacS*/*gacA* system. External signals are recognized by the histidine kinase GacS, which phosphorylates the response regulator GacA that indirectly influences quorum sensing processes. IM is inner membrane, OM is outer membrane. Arrows represent crosstalk between the various system. Adapted from Rutherford and Bassler 2012 and Song, Li, and Wang 2023. (B-C) The fraction of individuals GFP positive following exposure to exogenous Orsay virus. Data are from a single representative experiment, bars represent mean, dots represent individual plates. (B) Wild-type *P. aeruginosa* PA01 compared to mutant *P. aeruginosa* PA01 strains using 10a.u. of Orsay virus. (C) Wild-type *P. aeruginosa* PA14 compared to mutant *P. aeruginosa* PA14 strains using 100a.u. of Orsay virus. (D) Incidence proportion of Orsay virus transmission in the presence of *P. aeruginosa* wild-type versus select *P. aeruginosa* mutants. Data are from three experiments, bars represent mean, dots represent individual plates. For all plots error bars represent 95% C.I. For B-E, p-values were determined using one-way ANOVA followed by Dunnett's test. (NS non-significant, *p<0.05, **p<0.01, ***p<0.001).

269 ***P. lurida gacA* is required for attenuation of Orsay virus transmission.**

270 We next sought to determine whether our findings from the interaction of *C. elegans* and
271 Orsay virus in the presence of *P. aeruginosa*, a well-characterized pathogen of *C. elegans*, could
272 inform us further regarding the mechanisms underlying the attenuation of Orsay virus
273 transmission in the presence of *P. lurida* MYb11, a non-pathogenic constituent of the *C. elegans*
274 microbiota[39]. In particular, we identified a *gacA* ortholog using Orthovenn2 and generated a
275 putative knockout allele by removing the central 194 out of 214 amino acids (Fig. 5A)[54]. Following
276 exposure to exogenous Orsay virus, knockout of *gacA* in *P. lurida* MYb11 led to infection of 80%
277 of the population compared to infection of only 14% of the population in the presence of wild-
278 type *P. lurida* MYb11 from exogenous Orsay virus (Fig. 5B). The *gacA* mutation also suppressed
279 the attenuation of Orsay virus transmission, increasing the incidence proportion 2.9-fold
280 compared to wild-type *P. lurida* MYb11 (Fig. 5C). These data suggest that *gacA* has a conserved
281 role across distant *Pseudomonas* species in the attenuation of Orsay virus transmission and
282 infection of *C. elegans*.

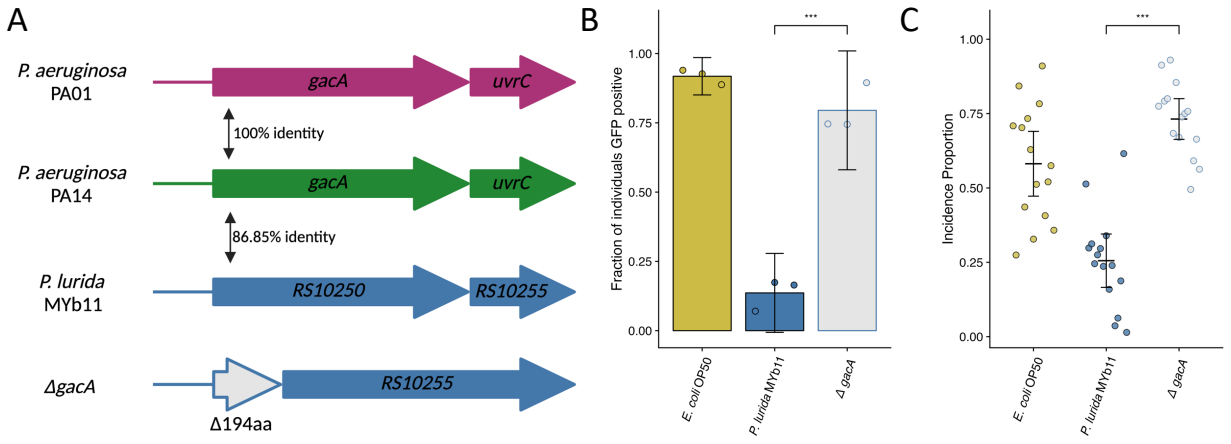

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Figure 5: Mutation of *P. lurida* MYb11 *gacA* suppresses the attenuation of Orsay virus infection.
(A) Diagram depicting *P. aeruginosa* PA01, *P. aeruginosa* PA14, and *P. lurida* MYb11 *gacA* as well as the *P. lurida* MYb11 *gacA* deletion mutant. Percent identity of the encoded protein was assessed using Clustal Omega. The *gacA* deletion removed 194 amino acids from the protein leaving 10 amino acids from both the N and C termini. Schematics made using Biorender.(B) The fraction of individuals GFP positive following exposure to 10a.u. exogenous Orsay virus in the presence of wild-type *P. lurida* MYb11 compared to a *gacA* mutant. Data are from a single representative experiment, bars represent mean, dots represent individual plates. (C) Incidence proportion of Orsay virus transmission in the presence of wild-type *P. lurida* MYb11 compared to a *gacA* mutant. Data are from three experiments, bars represent mean, dots represent individual plates. For all plots error bars represent 95% confidence interval (C.I.). p-values were determined using Student's t-Test (NS non-significant, *p<0.05, **p<0.01, ***p<0.001).

284

285 ***P. aeruginosa* genes linked to virulence reduce Orsay virus transmission and infection.**

286 We designed a candidate-based screen using a non-redundant transposon insertion
287 library to gain further insight into the genetic regulation of *P. aeruginosa* mediated reduction of
288 Orsay Virus transmission and infection (Fig. 6A)[55]. We identified candidate genes to include in our
289 screen from three sources: *gacA*-regulated genes, *rhlR*-regulated genes (but *rhlI*- or *pqsE*-
290 independent), and the set of genes required for full virulence in *C. elegans* previously identified
291 by Feinbaum et al. (Fig 6A)[47,56,57]. Of the 201 genes tested, 15 putative hits were identified with
292 the corresponding *P. aeruginosa* PA14 mutants exhibiting suppression of the attenuation of
293 Orsay virus infection by wild-type *P. aeruginosa* PA14 (Fig. 6A, Supp. Table 3). Using these 15
294 candidate genes, we performed an additional set of susceptibility assays which confirmed six of
295 the hits (Fig. 6B, Supp. Table 3). The identified hits grouped into two clusters based on the
296 strength of their suppression. Transposon insertion in the genes *ptsP*, *prpC*, and *kinB* led to
297 marked suppression of the attenuation of Orsay Virus leading to infection of 97%, 89%, and 82%
298 of the population respectively compared to only 4.2% of the population in the presence of wild-
299 type *P. aeruginosa* PA14. Transposon insertion in three additional genes, *clpA*, *glnK*, and *fabF1*
300 resulted in weaker, but robust suppression of the attenuation of Orsay virus leading to infection
301 of 34%, 28%, and 17% of the population respectively. We additionally tested whether transposon
302 insertion into these genes suppressed attenuation of Orsay virus transmission. While we
303 observed a trend towards mutations affecting susceptibility also affecting virus transmission, we

304 again observed a high degree of variation in the transmission assay, such that only mutation of
305 *ptsP* led to statistically significant suppression of Orsay virus attenuation (Fig. 6C). However,
306 similar to the effect observed with *lasI* mutation, the distributions of the incidence proportion
307 observed in the presence of the *prpC*, *kinB*, or *glnK* mutants were qualitatively different from the
308 nearly uniform incidence proportion observed in the presence of wild-type *P. aeruginosa* PA14
309 suggesting that mutation of these genes was impacting the attenuation of Orsay virus
310 transmission to some extent (Fig. 6C).

311 All six of the hits originated from the set of genes required for full *P. aeruginosa* PA14
312 virulence in *C. elegans*. We noted that these hits represented only six out of the 41 tested genes
313 that are known to influence virulence, indicative of some degree of specificity in the
314 consequences of these mutations beyond their general effect on *P. aeruginosa* PA14 virulence[57].
315 While these six genes have orthologs within *P. lurida* MYb11, knockout of the *P. lurida* MYb11
316 orthologs of *ptsP*, *prpC*, or *kinB* failed to suppress the attenuation of Orsay virus infection or
317 transmission by *P. lurida* MYb11(Supp. Fig. 2A and 2B)[57,58]. One explanation of these results is
318 that these genes play different roles within *P. lurida* MYb11 and therefore their mutation did not
319 have the same consequences for Orsay virus attenuation. Alternatively, these results might
320 further suggest that *ptsP*, *prpC*, and *kinB* act to attenuate Orsay virus infection via some specific
321 effect on *P. aeruginosa* PA14 virulence.

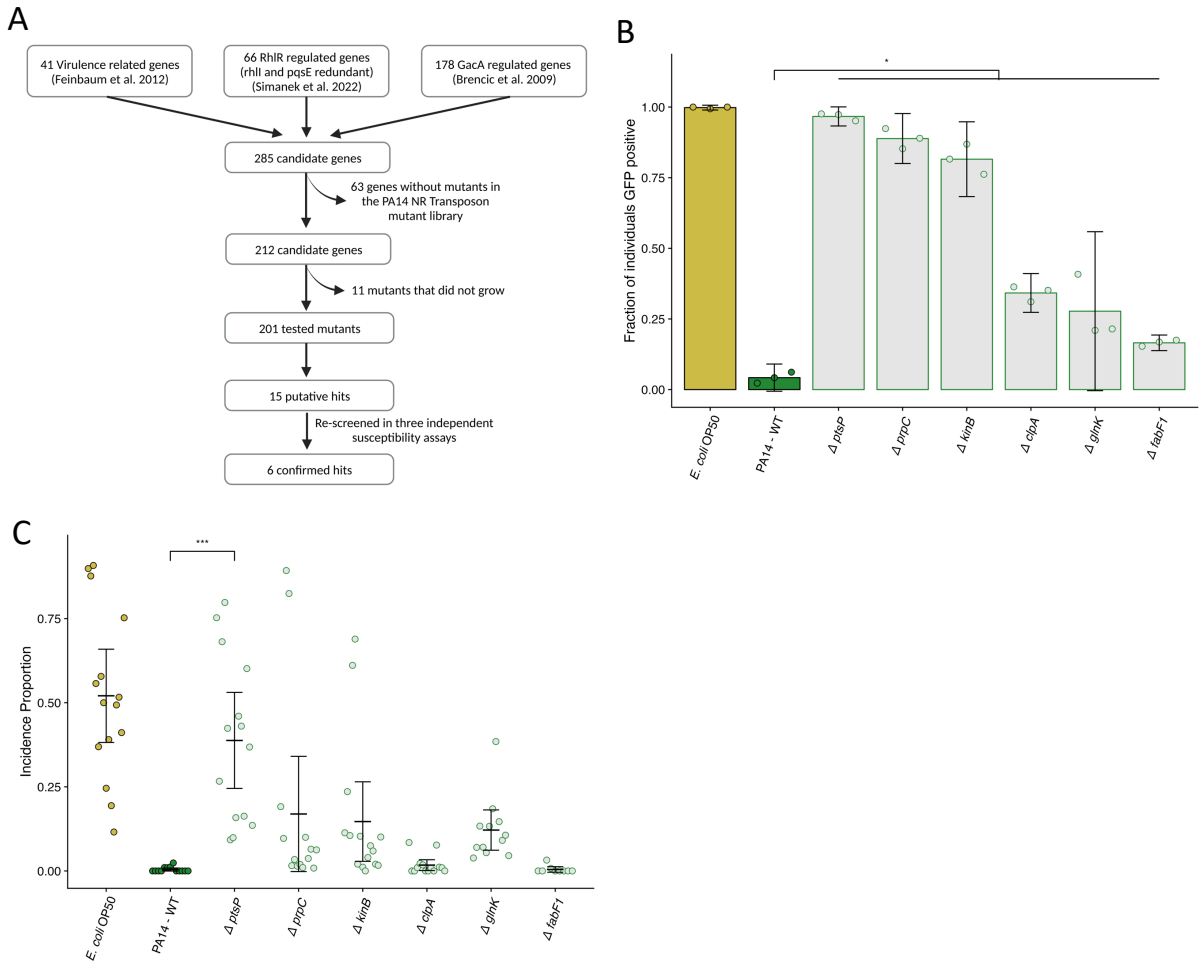


Figure 6: Mutation of *P. aeruginosa* PA14 genes related to virulence suppresses the attenuation of Orsay virus infection.
(A) Diagram describing the design and results of the screen to identify suppressors of *P. aeruginosa* PA14 Orsay virus attenuation. (B) The fraction of individuals GFP positive following exposure to 100 a.u. of exogenous Orsay virus. Data are from a single representative experiment. (C) Incidence proportion of Orsay virus transmission while individuals are present on *P. aeruginosa* PA14 wild-type compared to *P. aeruginosa* PA14 mutants. Data are from three experiments and dots represent individual plates. For all plots the black bar is the mean and error bars are the 95% confidence interval (C.I.). p-values determined using one-way ANOVA followed by Dunnett's test (NS non-significant, *p<0.05, **p<0.01, ***p<0.001).

Discussion

In our study, we quantitatively define the effect of bacteria on the transmission of Orsay virus in the *C. elegans* host. While the presence of *O. vermis* MYb71 enhanced the transmission of Orsay virus, the presence of *P. lurida* MYb11 or *P. aeruginosa* strains PA01 and PA14 attenuated Orsay virus transmission. The enhancement of Orsay virus transmission by *O. vermis* MYb71 and reduction of transmission by *P. lurida* MYb11 and *P. aeruginosa* PA01 and PA14 was mirrored in assays assessing infection rates by exogenous virus. Our results using exogenous virus demonstrate that host susceptibility to Orsay virus infection may vary by over three orders of

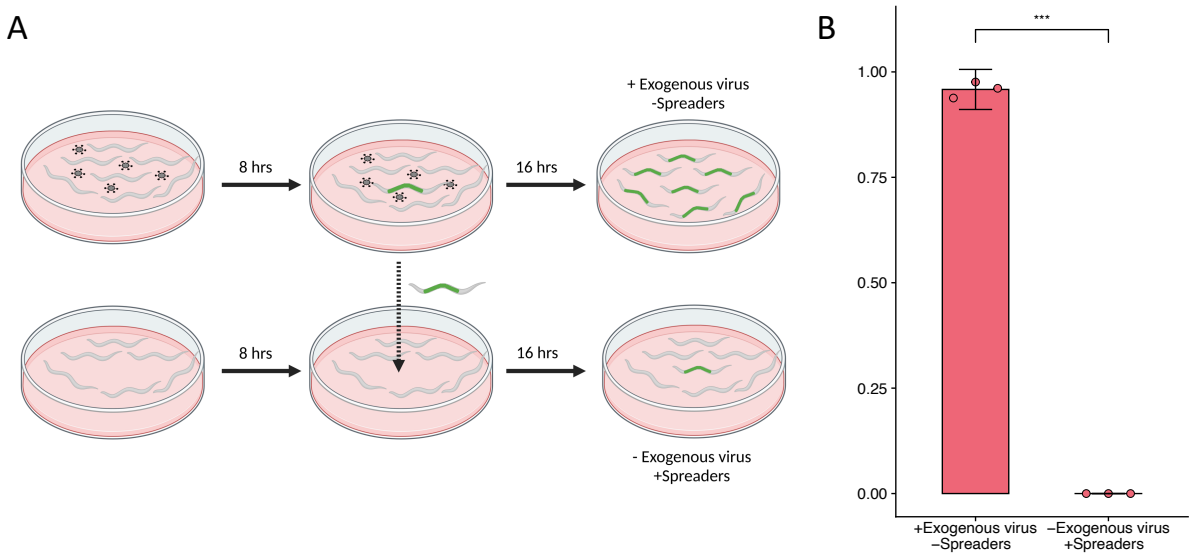
332 magnitude in the presence of *O. vermis* MYb71 versus *P. lurida* MYb11 which are found in the
333 natural environment in association with *C. elegans*. Moreover, we observe that pathogenic *P.*
334 *aeruginosa*, which may be found in natural environments, can further attenuate Orsay virus
335 transmission[31]. Our work is also consistent with a recent study by González and Félix that also
336 reported that monoaxenic cultures of other bacteria from the environment of *C. elegans* impact
337 host susceptibility[59].

338 Our observations that two *Ochrobactrum* species promoted transmission of Orsay virus
339 are intriguing given that other *Ochrobactrum* species have also been linked to viral infections.
340 *Ochrobactrum intermedium* promoted poliovirus infection in mice and enhanced poliovirus
341 stability in vitro[13]. *Ochrobactrum anthropi*, an opportunistic human pathogen, was identified
342 using a random forest analysis as one of the most predictive features differentiating the upper
343 respiratory tract of human patients recovering from influenza infection versus healthy controls[60].
344 Our observations in the *C. elegans* host raise the speculative possibility that *Ochrobactrum*
345 colonization may have roles in evolutionarily diverse hosts in modulating viral infection.

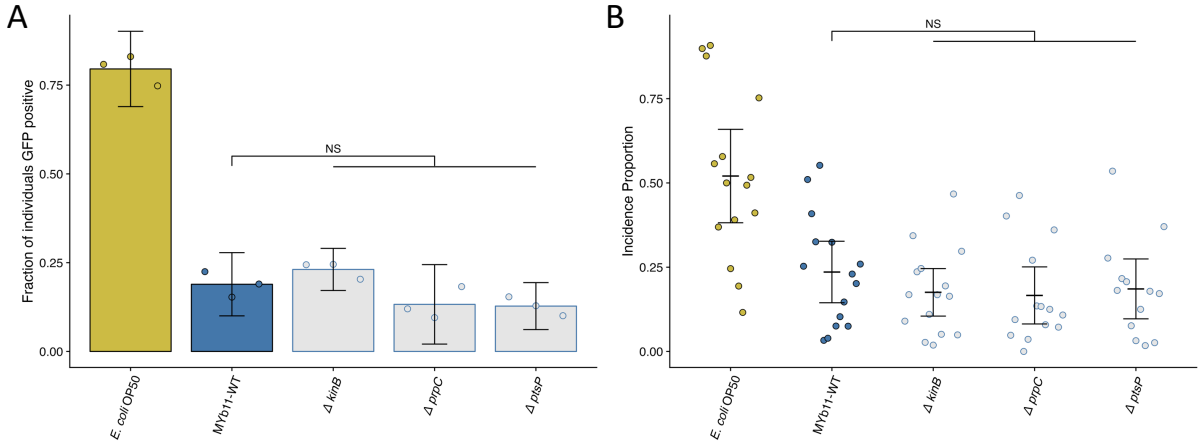
346 *P. lurida* MYb11 and *P. aeruginosa* PA01 and PA14 shared the ability to attenuate Orsay
347 virus infection and transmission, even as *P. lurida* and *P. aeruginosa* have markedly distinct
348 effects on the *C. elegans* host. *P. lurida* MYb11 promotes developmental rate and fitness,
349 although may do so at a minor cost to overall lifespan[39,61]. In contrast, *P. aeruginosa* is a pathogen
350 of *C. elegans* and numerous other organisms[30,62]. We identified that *gacA* regulates the
351 attenuation of Orsay virus by *P. aeruginosa* PA01 and PA14 and *P. lurida* MYb11. The *gacS/gacA*
352 system regulates many phenotypes in a variety of γ-proteobacteria[63]. In Pseudomonads,
353 *gacA/gacS* regulate processes related to quorum sensing, virulence, and biocontrol in a

354 population density dependent manner[63]. These data raise the possibility that *Pseudomonas*
355 quorum sensing pathways are required for the effects on Orsay virus transmission and infection.
356 Quorum sensing also influences additional aspects of *P. aeruginosa* physiology, including
357 swarming behaviors, biofilm formation, secondary metabolism rates, and overall transcription
358 patterns[44].To gain additional insights into the *P. aeruginosa* effects on Orsay virus transmission
359 we conducted a candidate-based screen including genes influenced by quorum sensing and genes
360 that regulate virulence towards *C. elegans*[47,56,57]. We identified six genes, *ptsP*, *prpC*, *kinB*, *clpA*,
361 *glnK*, and *fabF1,* that when mutated suppressed *P. aeruginosa* PA14 attenuation of Orsay virus
362 infection. Each of these genes has been shown to be required for full *P. aeruginosa* PA14
363 virulence in *C. elegans*[57]*.* However, we observed that only these six out of the total 41 virulence-
364 related genes identified in Feinbaum *et al*. influenced Orsay virus infection, arguing against a role
365 for general virulence in the attenuation of Orsay virus transmission and infection caused by *P.*
366 *aeruginosa* PA14 and *P. aeruginosa* PA01[57].

367 *C. elegans* is unlikely to associate with a single bacterium in its natural environment.
368 However, *C. elegans* shows clear behavioral preferences for grazing on certain bacteria from its
369 environment and may eat monoxenic lawns in the wild[64]. Our data demonstrate that individual
370 bacteria can have a profound impact on Orsay virus transmission rates in a species-specific
371 manner. Additionally, the tractability of the *C. elegans*-Orsay virus experimental system allowed
372 us to identify molecular determinants of viral transmission and will be useful for identifying
373 additional biotic factors that influence viral transmission. Our work builds on the expanding body
374 of knowledge showing that the microbiota can influence the interactions between viruses and
375 their animal hosts.



Supplementary Figure 1: Transmission does not occur from individuals infected during the course of a transmission or susceptibility assay.
(A-B) Individuals were exposed to 0 a.u. or 5 a.u. exogenous Orsay virus in the presence of *O. vermis* MYb71. 8 h post infection five infected individuals from the 5 a.u. plate were transferred to the 0 a.u. plate to assess whether transmission from these individuals would occur. Both plates were scored 16 h after the transfer. Data are from a single representative experiment, bars represent mean, dots represent individual plates. Error bars are the 95% confidence interval (C.I.). p-value determined by Student's t-test (NS non-significant, *p<0.05, **p<0.01, ***p<0.001).



Supplementary Figure 2: Knockout of the *P. lurida* MYb11 orthologs of *ptsP*, *prpC*, or *kinB* does not suppress Orsay virus attenuation in susceptibility or transmission assays.
(B) The fraction of individuals GFP positive following exposure to 10 a.u. of exogenous Orsay virus in the presence of the indicated bacteria. Data are from a single representative experiment. (C) Incidence proportion of Orsay virus transmission in the presence of the indicated bacteria. The *E. coli* OP50 reference is shared with Figure 5C. Data are from three experiments and dots represent individual plates. For all plots the black bar is the mean and error bars are the 95% confidence interval (C.I.). p-values determined using one-way ANOVA followed by Dunnett's test (NS non-significant, *p<0.05, **p<0.01, ***p<0.001).

383 **Materials and methods**
384

385 *C. elegans* strains and growth conditions

386 *C. elegans* were maintained on NGM agar plates (17 g agar, 2.5 g peptone, 3 g NaCl per 1 L water)
387 containing *E. coli* OP50[41]. Strains bearing the *glp-4(bn2)* temperature sensitive mutation were
388 maintained at 16°C, while all other were maintained at 20°C. All assays were performed on SKA
389 assay plates at 20°C (17 g agar, 3.5 g peptone, 3 g NaCl per 1 L water)[30]. A full list of *C. elegans*
390 strains used in this study is contained within the Supplement (Supplement Table 1). For all assays
391 relying upon *pals-5p::GFP*, induction was manually assessed using a Nikon SMZ18
392 stereofluorescent microscope.
393

394 Bacterial strains and growth conditions

395 All bacteria were grown in Luria broth (10 g tryptone, 5 g yeast, 10 g NaCl per 1 L water). *E. coli*
396 OP50 and *P. aeruginosa* strains were grown at 37°C with shaking, while all other strains were
397 grown at 27°C with shaking. A full list of bacterial strains used in this study is contained within
398 the Supplemental Information (Supplement Table 2).
399

400 Orsay virus isolation and dose testing

401 Orsay virus was isolated from infected WUM31(*rde-1(ne219);jyIs9[pals-5p::gfp;myo-*
402 *2p::mCherry]*) individuals. Plates with gravid WUM31 were bleached to obtain eggs. Eggs were
403 hatched overnight in M9 solution rotating at 20°C and the L1 larvae were arrested to synchronize
404 the population. L1 larvae were combined with 100 μL of 6x concentrated *E. coli* OP50 and 50 μL

405 of Orsay virus filtrate, plated on SKA plates, and once dried, placed at 20°C for 48 h. Four GFP
406 positive individuals were then transferred to a new 6cm NGM plate with *E. coli* OP50 and
407 maintained until just starved. Twenty such 6cm plates were washed with 10 mL of M9 and the
408 resulting suspension was Dounce homogenized. Alternatively, 3.5 cm SKA plates containing
409 infected WUM31 animals was allowed to starve and then equally chunked onto four 10 cm NGM
410 plates with *E coli* OP50. Once these plates were just starved, eight 10 cm plates were washed and
411 homogenized as above. The homogenized suspension was then centrifuged at 13.2xg for five
412 min. The supernatant was passed through a 0.22 μm filter and aliquoted. Each batch of virus was
413 tested for potency and an ID_{50} calculated for *E. coli* OP50. Doses referenced throughout the
414 manuscript refer to actual microliters applied to each plate. However, no method was used to
415 normalize doses between batches of virus and so we have chosen to refer to doses in terms of
416 arbitrary units (a.u.). The average ID_{50} while on *E. coli* OP50 for all virus batches used in this study
417 was 3.6 suggesting an average dose of 1 a.u. roughly corresponds to 0.28x the ID_{50} on *Escherichia*
418 *coli*.
419
420 <u>Preparation of Uninfected Individuals for transmission and susceptibility assays</u>
421 Prior to all assays, plates with fecund ZD2611(*glp-4(bn-2);jyIs8[pals-5p::gfp;myo-2p::mCherry]*)
422 were bleached to obtain eggs. Eggs were hatched overnight in M9 solution rotating at 20°C and
423 the L1 larvae were arrested to synchronize the population. ZD2611 L1s were dropped onto plates
424 containing *E. coli* OP50 and placed at 20°C for 64-72 h. At this temperature reproduction is
425 delayed, but not eliminated. The resulting young adults were washed off the plates in M9 and
426 centrifuged at 1000xg for one minute. After removing the supernatant, the young adults were

427 once again washed in M9, then centrifuged at 1000 x g for one minute. Young adults were directly
428 dropped onto prepared plates described below.
429
430 Preparation of Bacteria for transmission and susceptibility assays
431 Unless otherwise indicated, bacteria were cultured overnight and added to cover the assay
432 plates. Plates containing *E. coli* OP50 or *P. aeruginosa* strains were placed at 37°C for 24 h. Plates
433 containing *O. vermis* MYb71 or *P. lurida* MYb11 strains were placed at 25°C for 24 h. All plates
434 were then moved to room temperature for an additional 24 h before the addition of virus.
435
436 For the natural isolate transmission screen and early transmission assay cultures were grown to
437 late stationary phase. Cultures were then spun down for six minutes at 4000xg and reconstituted
438 to 25 mg/mL in their own supernatant. 150 μL of this suspension was combined with 50 μL of a
439 mix of M9 for transmission assays or 50 μL of a combination of Orsay virus filtrate and M9 for the
440 early transmission assay. This mixture was combined with young adult ZD2611 individuals and
441 directly added to assay plates before drying.
442
443 Transmission assays
444 To obtain infected spreader animals, fecund ZD2610 (*glp-4(bn-20);rde-1(ne219); jyIs8[pals-*
445 *5p::gfp;myo-2p::mCherry]*) were bleached to obtain eggs. Eggs were hatched overnight in M9
446 solution rotating at 20°C and the L1 larvae were arrested to synchronize the population. Arrested
447 ZD2610 L1s were dropped onto NGM plates containing *E. coli* OP50 and placed at 20°C for 48 h.
448 Animals were then washed off the plates in M9 and centrifuged at 1000xg for one min. After

449 removing the supernatant, the young adults were washed with M9 again then centrifuged at
450 1000xg for one min. Supernatant was once again removed, and individuals were immediately
451 mixed with 100 μL of 6x overnight *E. coli* OP50 culture and 50 μL of Orsay virus for a minimum of
452 18-20 h. GFP-positive ZD2610 young adults were identified and picked onto transfer plates
453 containing the appropriate bacteria prepared as indicated above for 4-6 h. After this period, five
454 spreaders were transferred to assay plates containing the same bacteria and approximately 100
455 uninfected ZD2611 individuals to start the assay. Transmission assays were scored 24 h after
456 adding spreader individuals.
457
458 For the natural isolate transmission screen, arrested ZD2610 L1s were combined with 100 μL of
459 6x concentrated *E. coli* OP50 and 50 μL of Orsay virus filtrate and once dried, placed at 20°C for
460 64-72 h. On the day of the assay, GFP-positive ZD2610 young adults were identified and picked
461 onto transfer plates containing the appropriate bacteria prepared as indicated above for 4-6 h.
462 After this period, five spreaders were transferred to assay plates containing the same bacteria
463 and approximately 100 uninfected ZD2611 individuals to start the assay.
464
465 <u>Incidence proportion</u>
466 Incidence proportion was calculated by dividing the number of newly infected animals by the
467 total non-spreader population per plate. Individual plates for which the total number of GFP
468 positive individuals after 24 h was less than the initial number of spreaders placed on the plate
469 were excluded.
470

471 Susceptibility assays

472 Unless otherwise indicated, Orsay virus filtrate at the indicated doses was diluted in filtered M9
473 solution. 200 uL total was applied to each assay plate and swirled to cover the entire bacterial
474 lawn. Plates were then dried before the addition of approximately 100 uninfected ZD2611
475 individuals prepared as indicated above. Assays were scored 24 h later using a Nikon SMZ18
476 stereo fluorescent microscope and the fraction of GFP-positive individuals was calculated.

477

478 Early Transmission Assay

479 For early transmission assay, young ZD2611 adults were exposed to 0a.u. or 5a.u. exogenous
480 Orsay virus in the presence of *O. vermis* MYb71. 8 hours post infection five infected individuals
481 from the 5 a.u. plate were transferred to the 0 a.u. plate to assess whether transmission from
482 these individuals would occur. Both plates were scored 16 hours after the transfer.

483

484 Fluorescence *in situ* hybridization

485 Previously published probes were obtained to target the RNA1 segment of Orsay virus[25]. Animals
486 were infected according to the methods described above for susceptibility assays. Animals were
487 processed according to the Stellaris RNA FISH Protocol for *C. elegans* (LGC Biosearch
488 Technologies) with minor modifications. Briefly, young adults were washed off the plate and
489 rinsed twice in M9. Animals were then fixed for 30 min rotating in a microcentrifuge tube at 20°C.
490 After washing twice with 1 mL of phosphate buffered saline animals were permeabilized in 70%
491 of ethanol and stored at 4°C for 1-7 d. Animals were washed with Wash Buffer A before addition
492 of 100 μL of hybridization buffer containing 3 μL of RNA1 probe mix. Probe was hybridized

493 overnight at 46°C. Animals were then washed with Wash Buffer A alone once, and then again
494 with Wash Buffer A containing 5 ng/mL DAPI. Lastly 100 μL of Wash Buffer B was added before
495 mounting animals on slides with 25 μL of Vectashield Mounting Medium. The fraction of
496 individuals with RNA1 staining was then quantified using a Nikon SMZ18 stereofluorescent
497 microscope or a Zeiss AxioImager Z1 compound fluorescent microscope.
498
499 RNA Extraction
500 Animals were washed 5 times in M9 and collected in TRIzol reagent (Invitrogen) and stored at -
501 80C before extraction. RNA extraction was performed using Direct-zol RNA microprep kits
502 (Zymo Research) following the manufacturer's instructions.
503
504 qPCR
505 cDNA was made using 500ng of RNA as template (Promega GoScript Reverse
506 Transcriptase/Random Primers). cDNA was diluted at 1/40 and qPCR were performed using 1ul
507 of diluted cDNA (GoTaq Promega) and run on QuantStudio 3 Real Time PCR system. RNA1 levels
508 were then quantified via qPCR using previously published primers[23].
509
510 Plasmid-based replication experiments
511 Adult animals carrying a transgene containing the wild-type RNA1 or RNA1D601A Orsay virus
512 genome segment under the control of a heat-inducible promoter were placed on the indicated
513 bacteria for 4 h before heat-shock at 33°C for 2 h[43]. Animals then recovered at 20°C for 20 h

514 before harvesting for RNA extraction and qPCR as detailed above. RNA1 levels were normalized
515 to the values obtained from animals bearing the wild-type RNA1 and exposed to *E. coli* OP50.
516
517 Orsay virus replication in the presence of *P. aeruginosa* PA14
518 Adult ERT54 (*jyIs8[pals-5p::gfp;myo-2p::mCherry])* or WUM31 (*rde-1(ne219); jyIs8[pals-*
519 *5p::gfp;myo-2p::mCherry)]* were exposed to exogenous Orsay virus in the presence of *P.*
520 *aeruginosa* PA14. After 2 h or 24 h, animals were harvested for RNA extraction and qPCR as
521 detailed above. RNA1 levels were quantified via qPCR. Within each genotype, the data for each
522 experiment were normalized to the 2 h timepoint.
523
524 *Pseudomonas lurida* MYb11 mutant construction
525 A protocol developed for allelic exchange in *P. aeruginosa* was modified for use in *P. lurida*
526 MYb11[65]. Briefly, homology arms flanking the region to be deleted were obtained using
527 polymerase chain reaction (PCR) and cloned into the pExG2-KanR suicide vector using Hi-Fi
528 Assembly (New England Biolabs)[66]. DH5α *E. coli* were transformed using a standard heat shock
529 protocol. Successful transformants were selected for on LB+Kanamycin (50 μg/mL) plates and
530 colony PCR was performed to check for proper insert size in the transformants. *E. coli* bearing
531 the desired plasmid were grown overnight in LB+Kanamycin (50 μg/mL). Plasmids were then
532 obtained using a Qiagen MiniPrep Kit (Qiagen). Plasmids were assessed for the desired sequence
533 by Sanger sequencing and transformed into *P. lurida* MYb11 using the following electroporation
534 procedure. *P. lurida* MYb11 was grown overnight then placed on ice for 30 min. The culture was
535 spun down at 4°C and washed twice with ice-cold water. After reconstitution in 100 μL of ice-

536 cold water the suspension was transferred to a pre-chilled cuvette and transformed at 630 kV
537 using an Eporator (Eppendorf). 900 μL of LB was added, and the suspension was transferred to a
538 microcentrifuge tube and incubated at 27°C for 2 h with shaking. The suspension was then plated
539 on LB+Kanamycin (50 μg/mL) plates and grown for 48 h at 25 °C. Colonies were picked and grown
540 overnight in LB. Cultures were streaked onto sucrose plates (15 g agar, 10 g tryptone, 5 g yeast,
541 60 g sucrose per 1 L water) to perform sucrose-based counter selection[65]. Colonies that survived
542 were genotyped for the expected deletion.
543
544 Data visualization and statistics
545 All experiments were performed three times. For transmission, qPCR, and FISH-based
546 susceptibility assays data from each experiment are combined. For susceptibility assays a single
547 representative experiment is shown. Data were analyzed in R Studio[67]. When comparing all the
548 means of more than two groups p-values were calculated using one-way ANOVA followed by the
549 Tukey HSD test. When comparing multiple experimental groups to a control group p-values were
550 calculated using one-way ANOVA followed by Dunnett's test. P-values for assays comparing only
551 two groups were calculated using Student's t-test or Welch's t-test as indicated. *E. coli* OP50 is
552 included in all experiments as a reference but was not included for statistical comparison unless
553 explicitely noted. Susceptibility assay curves were modeled using the drc[68] package in R. A two-
554 parameter log-logistic function was used to model the curve and ID_{50} values were calculated
555 using the ED function. Plots were made using the gdata[69], scales[70], drc[68], Rmisc[71], multcomp[72],
556 ggplot2[73], ggsignif[74], and cowplot[75] packages.
557

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568

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