Microbiology and Infectious Disease

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 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.

eLife assessment

Using a *C. elegans*/virus system, this **important** work demonstrates that viral susceptibility can be greatly altered by the bacterial food that *C. elegans* consumes. The work is rigorous with **solid** support for the conclusions: the authors show that quorum-sensing compounds play a role in reducing host susceptibility, and they perform control experiments to rule out nutrition and pathogenicity of the bacteria as the cause of impacts on viral susceptibility.

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Introduction

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

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

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

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

In this study, we sought to understand how bacteria that are constituents of the *C. elegans* microbiota quantitatively affect the transmission of Orsay virus in *C. elegans.* We observed that monoaxenic cultures of different bacterial species had widely divergent effects on the transmission of Orsay virus and conducted genetic analysis of the bacterial determinants involved in modulating virus transmission. Our data point to a key species-specific role for bacteria as critical determinant of virus transmission.

Results

Wide variation in the effects of bacteria on the transmission of Orsay virus

Orsay virus spreads horizontally through the fecal-oral route and transmission can spread from a single animal to a population of animals on a plate[40]. We sought to assess the impact of bacteria on Orsay virus transmission rates and utilized a collection of bacteria isolated from the environment with wild *C. elegans* to assemble a panel of Gram-negative bacteria for comparison with the standard laboratory bacterial food, *Escherichia coli* OP50[36,37,39,41,42]. All animals were raised to young adulthood on lawns of *E. coli* OP50 to eliminate differences in developmental rates caused by different bacteria[39]. We set up a transmission assay by placing infected animals ("spreaders") together with uninfected animals on a monoaxenic lawn of each test bacterium. We quantified the incidence proportion, defined as the number of new infections produced after 24 h as determined by detection of induction of the *pals-5p::GFP* reporter, which is induced by infection with Orsay virus (**Fig. 1A-C**)[27]. We observed a wide range in the measured incidence proportion (**Fig. 1B** and **1C**). Exposure to many of the naturally associated bacterial strains resulted in transmission that was comparable to the incidence proportion observed with *E. coli* OP50 with some prominent exceptions (**Fig. 1B** and **1C**). Two *Ochrobactrum* species promoted virus infection in nearly all individuals in the transmission assay and increased the incidence proportion 2.7-fold and 2.9-fold compared to *E. coli* OP50 (**Fig. 1B** and **1C**). We confirmed that transmission from the initial spreader animals in the assay, and not multiple rounds of infection, was responsible for the increased incidence proportion observed in the presence of *Ochrobactrum vermis* MYb71 (Supp. Fig. 1A-D). On the other hand, the presence of *P. lurida* MYb11 reduced the incidence proportion 4.1-fold compared to *E. coli* OP50 and 11-fold compared to *O. vermis* MYb71 (**Fig. 1B and C**). These results present a striking divergence in the effects of individual bacterial constituents of the *C. elegans* microbiota on the level of transmission of Orsay virus from infected animals.

We reasoned that increased transmission of Orsay virus could be due to bacterial modulation of host susceptibility to viral infection. To quantify the degree of host susceptibility to Orsay virus we added Orsay virus in doses that ranged across four orders of magnitude and then measured the fraction of individuals that became *pals-5p::GFP* positive after 24 h (**Fig. 1A** and **1D**, Supp. Fig. 2 and Supp. Fig. 3). We also specifically calculated the ID_{50}, or the dose of Orsay virus required to infect 50% of the population after 24 h of exposure (**Fig. 1D** and **1E**, Supp. Fig 2). On average, 3.6 µL of Orsay virus stock prepared as indicated in the Methods was required to infect 50% of a population of ZD2611 animals in the presence of *E. coli* OP50 after 24 hours (Supp. Fig. 2). We observed variability in the measured ID_{50} even when using the same batch of Orsay virus (e.g. **Fig. 1D** vs Supp. Fig. 3A vs. Supp. Fig. 3B). We therefore chose to control all our experiments internally rather than attempt to normalize between Orsay virus batches and we report doses used in arbitrary units (a.u.), however 1 a.u. corresponds to 1 µL of Orsay virus filtrate (Methods).

To determine quantitatively how different bacterial species modulate host susceptibility to viral infection independently of the potential differential effects of bacteria on host shedding of viruses, we added fixed doses of Orsay virus to plates of each bacterial species and monitored infection using the *pals-5p::GFP* reporter. At lower doses of Orsay virus, the presence of *O. vermis* MYb71 resulted in a higher fraction of infected animals than those observed in the presence of *E. coli* OP50 or *P. lurida* MYb11 (**Fig. 1D**, Supp. Fig. 3). At higher doses of Orsay virus, the presence of *P. lurida* MYb11 resulted in a reduced fraction of infected animals compared to *O. vermis* MYb71 and *E. coli* OP50 (**Fig. 1D**, Supp. Fig 3). We further quantified the ID_{50} in the presence of each bacterium and on average we observed that the ID_{50} in the presence of *E. coli OP50* was 14-fold

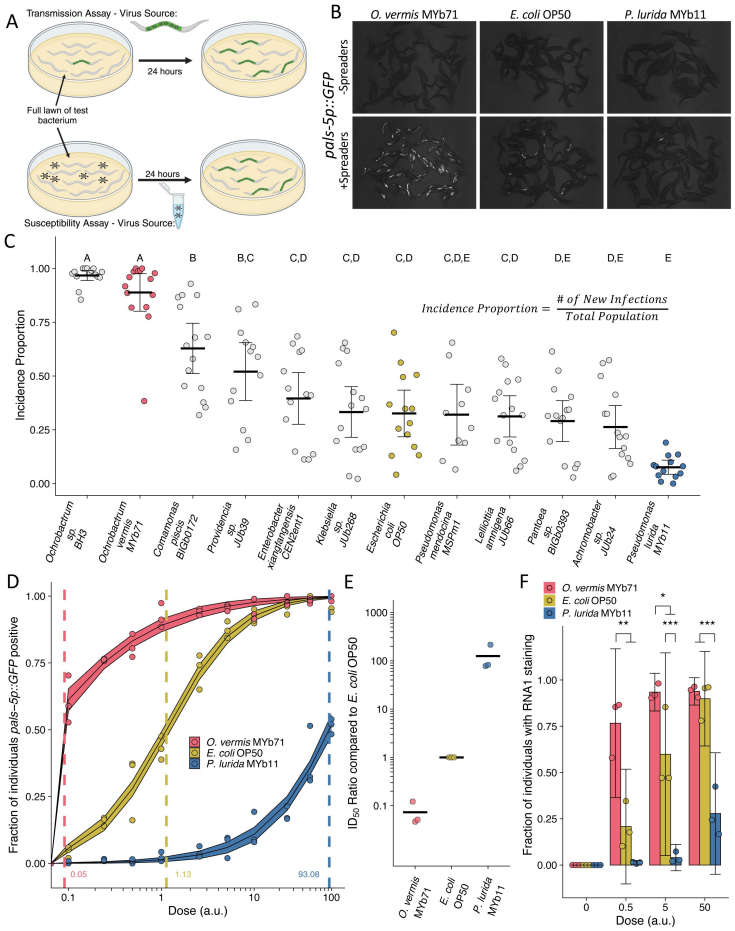

Figure 1

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 *pals-5p::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, and letters denote statistical significance. Treatments with the same letter are not significantly different from one another. (n=19694 in total and n > 42 for all dots). **(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 and replicates can be found in Supp. Fig. 3, Dots represent individual plates. ((a.u.), arbitrary units, see Methods) (n=8334 in total and n > 32 for all dots). **(E)** Ratios of the ID_{50} calculated on the indicated bacteria as compared to the ID_{50} calculated on *E. coli* OP50 from three separate experiments as in (D) and Supp. Fig. 3. **(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 replicate plates from the susceptibility assays in (D) and Supp. Fig. 3. (n=3446 in total and n > 61 for all dots). 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).

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higher than the ID_{50} in the presence of *O. vermis* MYb71 (**Fig. 1D** and **1E**, Supp. Fig. 3). The ID_{50} observed in the presence of *P. lurida* was 120-fold and 1800-fold higher than the ID_{50} in the presence of *E. coli* OP50 or *O. vermis* MYb71 respectively (**Fig. 1D** and **1E**, Supp. Fig. 3).

We corroborated our observations from the *pals-5p::GFP* reporter by scoring the same samples using fluorescence *in situ* hybridization to detect the RNA1 segment of the Orsay virus genome in the intestinal cells of infected animals (**Fig. 1F**). As expected, we observed that at lower doses the presence of *O. vermis* MYb71 resulted in a greater fraction of infected animals compared to *E. coli* OP50 or *P. lurida* MYb11, while at higher doses the presence of *P. lurida* MYb11 resulted in a reduced fraction of infected animals (**Fig. 1F**). Together these data establish that individual members of the *C. elegans* microbiota can modulate host susceptibility to Orsay virus over three orders of magnitude with dramatic consequences for the transmissibility of Orsay virus.

We also assessed whether bacteria altered host feeding behavior as this may influence the transmission and infection rate of Orsay virus. We quantified the number of pharyngeal pumps per minute (ppm), a metric of *C. elegans* feeding, in the presence of each bacterium. After 6 h of exposure to *E. coli* OP50, *P. lurida* MYb11, or *O. vermis* MYb71 there were no differences in pharyngeal pumping rates (Supp. Fig. 4A). At 24 h pumping rates were decreased in the presence of *O. vermis* MYb71 to an average of 242 ppm compared to 261 ppm in the presence of *P. lurida* MYb11 suggesting that changes to feeding behavior are unlikely to be responsible for observed effects on host susceptibility to Orsay virus (Supp. Fig. 4B).

P. aeruginosa attenuates Orsay virus transmission

In view of the effect of *P. lurida* MYb11 on attenuating Orsay virus infection of *C. elegans,* we examined the effect of the distantly related bacterium, *Pseudomonas aeruginosa,* an opportunistic pathogen of humans that has been characterized extensively[30,43]. We observed that in the presence of either *P. aeruginosa* strains PA01 or PA14, transmission from spreader animals to uninfected individuals was nearly completely blocked and the incidence proportion was 11-fold and 32-fold lower than that observed in the presence of *E. coli* OP50, respectively (**Fig. 2A**). The attenuating effect of *P. aeruginosa* PA01 and PA14 on virus transmission was further reduced 3.1-fold and 9.5-fold, respectively, compared to the incidence proportion observed in the presence of *P. lurida* MYb11 (**Fig. 2A**).

We confirmed that host susceptibility to Orsay virus was reduced in the presence of *P. aeruginosa* by performing susceptibility assays at two doses of exogenous Orsay virus (**Fig 2B**, Supp. Fig. 5). At each dose, fewer animals were infected following exposure to equivalent doses of Orsay virus in the presence of *P. aeruginosa* PA01, PA14, or *P. lurida* MYb11 as compared to the fraction of animals infected in the presence of *E. coli* OP50 (**Fig. 2B**, Supp. Fig. 5). At the highest dose of virus used, we still observed robust attenuation of infection in the presence of *P. aeruginosa* PA14 as 0.5% of the animals were infected as compared to 54%, 70%, and 97% in the presence of *P. aeruginosa* PA01, *P. lurida* MYb11, or *E. coli* OP50, respectively (**Fig. 2B**). We confirmed that no individuals were detectably infected with Orsay virus while in the presence of *P. aeruginosa* PA14 using FISH staining (**Fig. 2C**). FISH additionally confirmed that the presence of *P. aeruginosa* PA01 and *P. lurida* MYb11 attenuated average infection to 24% and 60% of the population respectively compared to *E. coli* OP50 which supported infection of 86% of the population. Further, *P. aeruginosa* PA01, *P. lurida* MYb11, and *E. coli* OP50 all supported higher levels of infection than *P. aeruginosa* PA14. (**Fig. 2C**). Together these results demonstrate a striking capacity of *P. lurida* MYb11 and *P. aeruginosa* strains to sharply reduce and even effectively block, host susceptibility to infection with Orsay virus.

We again assessed whether bacterial modulation of host feeding behavior may be responsible for the observed effects on host susceptibility to Orsay virus. There were no differences observed in pharyngeal pumping rate after 6 h of exposure to each bacterium (Supp. Fig. 4A). After 24 h pharyngeal pumping rate increased in the presence of *P. aeruginosa* PA14 to an average of 264

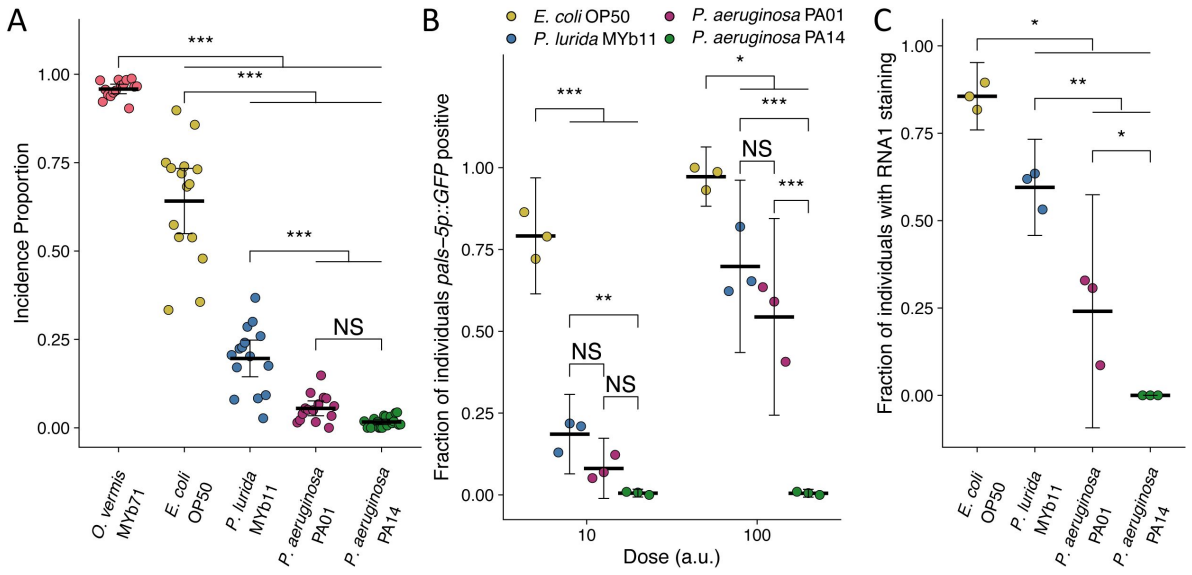

Figure 2

P. aeruginosa attenuates Orsay virus transmission and infection rates.

(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. (n=9214 in total and n > 54 for all dots) **(B)** The fraction of individuals *pals-5p::GFP* positive following exposure to two doses of exogenous Orsay virus. Data are from a single representative experiment and replicates can be found in Supp. Fig. 5. Dots represent individual plates. (n=6539 in total and n > 56 for all dots) **(C)** The fraction of individuals with staining following exposure to 100 a.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 replicate plates from (B) and Supp. Fig. 5. (n=2743 in total and n > 116 for all dots) 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).

ppm compared to 248 ppm in the presence of *E. coli* OP50 which suggests that changes to feeding behavior are unlikely to account for the attenuation of infection observed in *P. aeruginosa* (Supp. Fig. 4B).

P. aeruginosa is a pathogen of *C. elegans* and numerous other organisms[30,44,45]. Moreover, while both PA01 and PA14 are pathogenic, PA14 is more virulent towards *C. elegans*[30]. *P. lurida* MYb11 promotes developmental rate and fitness, although does so at a minor cost to overall lifespan[39,46]. The effect of O. vermis MYb71 on lifespan has not been assessed. We assessed host lifespan under our assay conditions to examine links between bacterial pathogenicity and host susceptibility to Orsay virus. Under our assay conditions, including a full lawn of bacteria, which prevent *C. elegans* from avoiding undesirable bacteria, and a temperature of 20°C, which is below the optimal virulence temperature of *P. aeruginosa,* substantial mortality is not observed until 72 hours after exposure (Supp. Fig. 6). All bacteria enhanced mortality in comparison to *E. coli* OP50 (Supp. Fig. 6). Interestingly, *O. vermis* MYb71 exposure led to similarly enhanced mortality when compared to *P. aeruginosa* PA14, and both bacteria enhanced mortality in comparison to *P. lurida* MYb11 or *P. aeruginosa* PA01 (Supp. Fig. 6). *P. lurida* MYb11 also enhanced mortality compared to *P. aeruginosa* PA01 under these conditions (Supp. Fig. 6). The similar lifespans of animals exposed to *P. aeruginosa* PA14 and *O. vermis* MYb71 stand in contrast to their divergent effects on host susceptibility to Orsay virus. This suggests that general pathogenicity is unlikely to be responsible for effects on host susceptibility, although pathogenic consequences specific to exposure to each bacterium may still contribute to the observed effects on host susceptibility to Orsay virus.

Attenuation of Orsay virus transmission by *P. lurida* and *P. aeruginosa* is not due to effects on Orsay virus replication, stability, or shedding

We considered the possibility that the attenuation of Orsay virus transmission in the presence of *P. lurida* and *P. aeruginosa* strains might be due to inhibitory effects of the bacteria on the replication of Orsay virus once transmitted to a susceptible animal host. To evaluate this possibility, we made use of a plasmid-based system in which viral RNA1 is expressed through a transgene introduced into *C. elegans,* so that replication of RNA1 can be assessed independent of the entry of exogenous virus into the host[47]. In this system, the Orsay virus RNA1 segment, which encodes the RNA-dependent RNA polymerase (RdRP), is expressed following heat-shock. The expressed RNA1 may then be translated to produce the RdRP which can then replicate RNA1 through a negative-strand intermediate. The expression of RNA1 via heat-shock bypasses any differences in viral entry or pre-replication steps, allowing for a direct test of RNA1 replication efficiency under different conditions[47]. A plasmid expressing a mutated RdRP (RNA1[D601A]) incapable of supporting further RNA1 replication after the initial heat-shock serves as a control for heat shock efficiency[47]. Using this system, we observed that Orsay virus RNA1 replication efficiency was unaffected by the presence of *Pseudomonas* species relative to RNA1 replication observed in the presence of *E. coli* OP50 (**Fig. 3A**). Additionally, there were no differences observed in heat-shock efficiency between the different bacteria (**Fig. 3A**). These data suggest that bacteria-induced differences in RNA1 replication in the host do not explain the substantial attenuation of Orsay virus transmission and infection rates caused by *P. aeruginosa* PA01 or PA14 and *P. lurida* MYb11.

We sought to confirm the successful replication of the plasmid expressed RNA1 of Orsay virus by assessing whether we could detect the replication of Orsay virus RNA1 in each *Pseudomonas* species. We exposed young adult wild-type (N2) or RNAi defective animals (*rde-1(ne219)*) to exogenous Orsay virus 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. Animals of the RNAi defective *rde-1(ne219)* background have a defective antiviral response leading to higher viral loads than wild-type animals[23]. The *rde-1(ne219)* mutant therefore provides a more

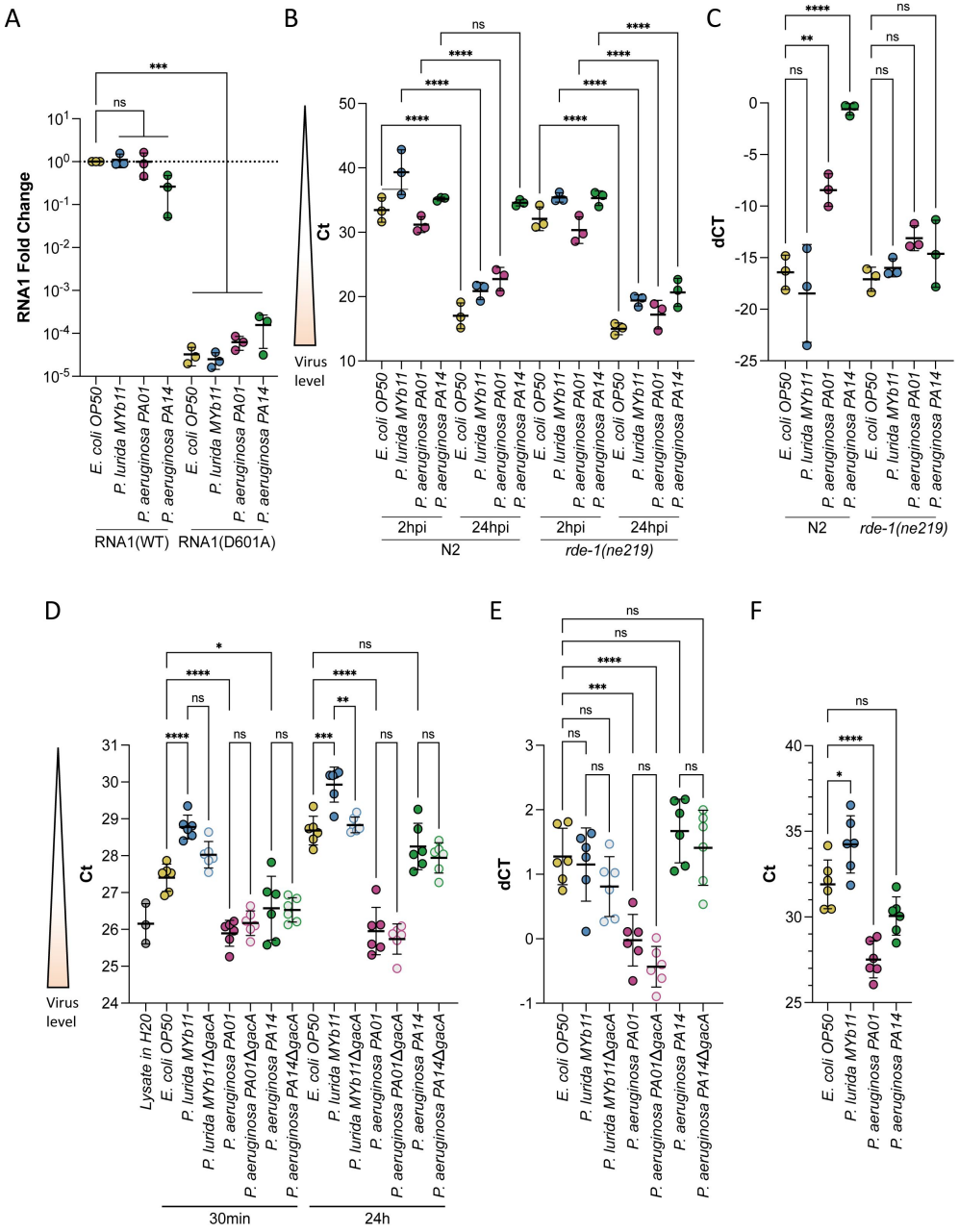

Figure 3

P. aeruginosa and *P. lurida* MYb11 do not eliminate Orsay virus replication, rapidly degrade Orsay virus, or eliminate Orsay virus shedding by infected spreader animals.

(A) Animals carrying either a wild-type (RNA1(WT)) or replication defective (RNA1(D601A)) transgenic viral RNA replicon system (Jiang et al. 2017) 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 the indicated bacterium 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. (**C**) Delta Ct comparing the 24 h and 2 h timepoints from (B). (**D**) Stability of Orsay virus in the presence of lawns of the indicated bacteria for the indicated time at 20°C. (**E**) Delta Ct comparing the 24 h and 30 min timepoints from (D). (**F**) Orsay virus RNA1 levels recovered from the indicated lawns after infected ZD2610(*rde-1(ne219);jyIs8[pals-5p::GFP;myo-2p::mCherry];glp-4(bn2ts)*) spreaders were allowed to shed for 24 hours mimicking a transmission assay. (NS non-significant, *p<0.05, **p<0.01, ***p<0.001).

sensitive genetic background in which to detect viral replication following rare infection events within a population as is expected in the presence of *P. aeruginosa* PA14. In *rde-1(ne219)* animals viral RNA1 levels increased at 24 h relative to 2 h post exposure regardless of the bacteria present (**Fig. 3B** and **3C**). On the other hand, in the wild-type background replication was not observed in animals exposed to *P. aeruginosa* PA14 and was attenuated in animals exposed to *P. aeruginosa* PA01 compared to animals exposed to *E. coli* OP50 or *P. lurida* MYb11 (**Fig. 3B** and **3C**). In a wild-type background individuals exposed to *P. aeruginosa* PA01 and *P. aeruginosa* PA14 are less likely to become infected from exogenous Orsay virus (**Fig. 2B** and Supp. Fig. 5). It is therefore likely that the viral load we observed in the wild-type populations is due to a smaller proportion of the population being infected, rather than hampered replication[23].

We also assessed whether *P. lurida* MYb11, *P. aeruginosa* PA01, or *P. aeruginosa* PA14 was capable of rapidly degrading Orsay virus or preventing viral shedding, either of which might account for attenuation of Orsay virus transmission. We did not observe a substantial decrease in viral stability in the presence of any bacterium suggesting Orsay virus is not rapidly degraded (**Fig. 3D** and **3E**). We did observe an approximately 2-fold decrease in viral levels between *E. coli* OP50 and *P. lurida* MYb11, however, this difference already existed within 30 min of exposure to each bacterium and did not grow further over the course of 24 h suggesting we are measuring differences in recovery of Orsay virus from each bacterial lawn, rather than enhanced degradation in the presence of *P. lurid*a MYb11 (**Fig. 3D** and **3E**). We measured rates of Orsay virus shedding during a transmission assay. We did not observe a substantial decrease in viral shedding in the presence of any bacterium (**Fig. 3F**). We observed approximately 4-fold lower levels of Orsay virus shed in the presence of *P. lurida* MYb11 vs *E. coli* OP50, but as suggested by our virus stability data, we believe these differences at least partly reflect differences in Orsay virus recovery from each bacterial lawn rather than actual differences in viral shedding (**Fig. 3F**).

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

Extensive studies on *Pseudomonas* species have demonstrated the importance of quorum sensing for regulating many community-level behaviors in response to growing population density[48]. *P. aeruginosa* relies upon three quorum sensing systems: *las*, *rhl*, and *pqs*. These systems are arranged hierarchically, however crosstalk between them is extensive (**Fig. 4A**)[48]. In *P. aeruginosa*, an additional layer of regulation stems from two-component regulatory systems such as *gacA/gacS* which regulates numerous genes that together influence quorum sensing, virulence, and biofilm development (**Fig. 4A**)[49–51]. *P. aeruginosa* possesses three exopolysaccharide biosynthetic clusters that each contribute to biofilm formation: *pel*, *psl*, and *alg*. However, *P. aeruginosa* PA01 preferentially produces Psl while *P. aeruginosa* PA14 is unable to synthesis Psl and produces Pel[52–56]. We hypothesized that quorum sensing might mediate the effect of *Pseudomonas* strains to attenuate virus transmission, while differences in exopolysaccharide production might mediate the enhanced attenuation of virus transmission observed in the presence of *P. aeruginosa* PA14 compared with what was observed in the presence of *P. aeruginosa* PA01. Therefore, we tested a panel of *P. aeruginosa* PA01 and *P. aeruginosa* PA14 quorum sensing and biofilm mutants to determine whether quorum sensing or biofilm formation was involved in the attenuation of Orsay virus infection mediated by *P. aeruginosa*.

Mutations of any the regulators of the *las*, *rhl*, or *pqs* quorum sensing systems suppressed the attenuation of Orsay virus infection caused by the presence of wild-type *P. aeruginosa* PA01 (**Fig. 4B**, Supp. Fig. 7). Knockout of *gacA* in *P. aeruginosa* PA01 also suppressed the attenuation of Orsay virus infection (**Fig. 4B**, Supp. Fig. 7). On the other hand, mutation of any of the three exopolysaccharide production pathways had no potent effect on the attenuation of infection (**Fig. 4B**, Supp. Fig. 7). These data support a role for quorum sensing regulated processes in reducing Orsay virus infection rates but do not implicate a role for individual exopolysaccharides.

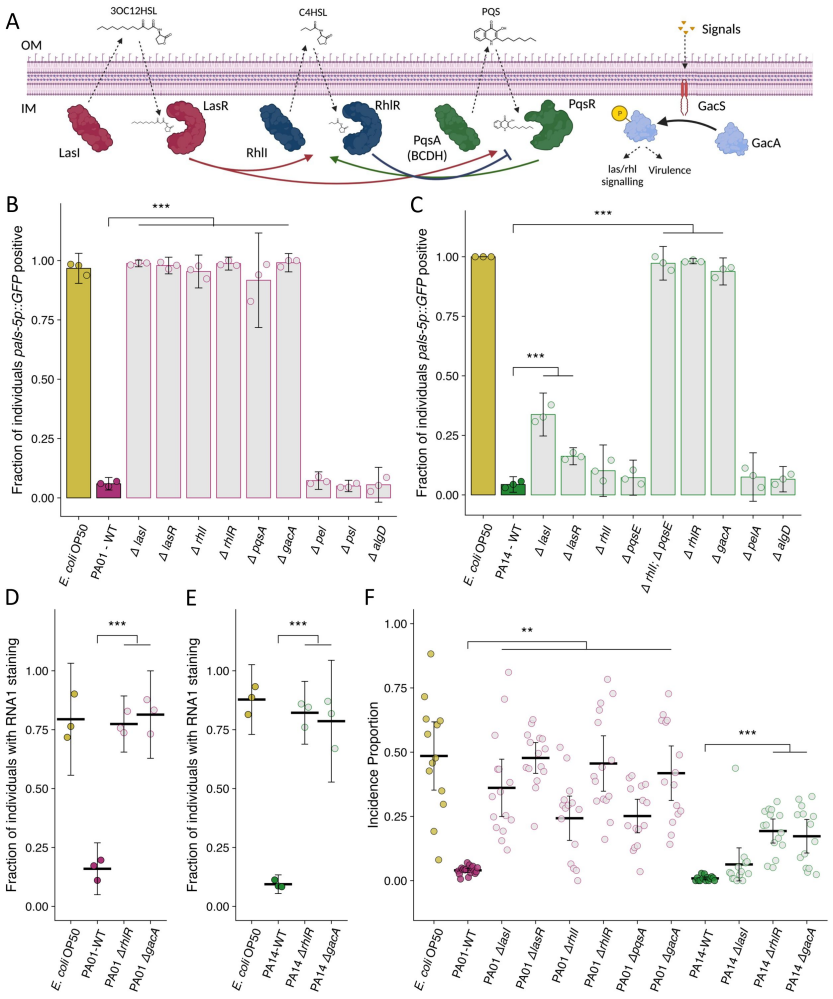

Figure 4

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 *pals-5p::GFP* positive following exposure to exogenous Orsay virus in the presence of the indicated bacterium. 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 10 a.u. of Orsay virus. Replicates can be found in Supp. Fig. 7. (n=4480 in total and n > 76 for all dots) **(C)** Wild-type *P. aeruginosa* PA14 compared to mutant *P. aeruginosa* PA14 strains using 100 a.u. of Orsay virus. Replicates can be found in Supp. Fig. 8. (n=3320 in total and n > 23 for all dots) **(D-E)** The fraction of individuals with staining following exposure to (D) 10 a.u. or (E) 100 a.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 a single experiment. ((D) n=1632 in total and n > 61 for all dots. (E) n=2000 in total and n > 60 for all dots) **(F)** 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. (n=20452 in total and n > 37 for all dots) 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).

For *P. aeruginosa* PA14, mutation of *gacA* or *rhlR* suppressed the attenuation of Orsay virus infection observed in the presence of wild-type *P. aeruginosa* PA14 (**Fig. 4C**, Supp. Fig. 8). Loss of the *rhlR* regulators *rhlI* or *pqsE* alone had no effect on the attenuation of Orsay virus infection, but simultaneous mutation of both *rhlI* and *pqsE* did suppress the attenuation of Orsay virus infection observed in the presence of wild-type *P. aeruginosa* PA14, similar to that observed for the *rhlR* mutant, suggesting *rhlI* and *pqsE* function redundantly to regulate *rhlR* in this context (**Fig. 4C**, Supp. Fig. 8)[57]. Mutation of *lasI* or *lasR* suppressed the attenuation of Orsay virus infection to a lesser extent (**Fig. 4C**, Supp. Fig. 8). Independent mutation of two genes responsible for *pel* or *alg* exopolysaccharide production had no effect on infection rates (**Fig. 4C**, Supp. Fig. 8). We further confirmed that mutation of *rhlR* and *gacA* in *P. aeruginosa* PA01 or *P. aeruginosa* PA14 suppressed the attenuation of Orsay virus infection via FISH (**Fig. 4D** and **4E**). These data suggest a role for quorum sensing in mediating *P. aeruginosa* PA14 suppression of Orsay virus infection, as we observed for *P. aeruginosa* PA01. However, our results obtained in the presence of *P. aeruginosa* PA14 suggest that there may be some differential regulation of the bacterial effectors responsible in comparison to *P. aeruginosa* PA01, or additional non-quorum sensing related factors that also mediate suppression.

As *P. aeruginosa* mutants could suppress the attenuation of infection observed by wild-type *P. aeruginosa* in the presence of exogenous virus, we next confirmed that these *P. aeruginosa* mutants similarly affected transmission from infected spreader animals. All *P. aeruginosa* PA01 quorum sensing mutants suppressed the attenuation of transmission by increasing the incidence proportion >6-fold compared to the wild-type *P. aeruginosa* PA01 (**Fig. 4F**). *P. aeruginosa* PA14 *rhlR* and *gacA* mutants suppressed the attenuation of transmission by increasing the incidence proportion 19-fold and 17-fold respectively compared to wild-type *P. aeruginosa* PA14, but a *lasI* mutant had minimal effect consistent with the pattern we observed in the susceptibility assay using exogenous Orsay virus (**Fig. 4F**). Likewise, the magnitude of suppression of the attenuation of Orsay virus transmission observed in the *P. aeruginosa rhlR* or *gacA* mutants was greater in the *P. aeruginosa* PA01 background compared to the *P. aeruginosa* PA14 background, potentially suggesting the existence of additional strain specific factors that act in *P. aeruginosa* PA14 to attenuate Orsay virus transmission.

Our findings suggest the possibility that quorum sensing molecules could act directly to attenuate infection. To explore this hypothesis, we prepared liquid *E. coli* OP50, *O. vermis* MYb71, *P. lurida* MYb11, *P. aeruginosa* PA01, and *P. aeruginosa* PA14 cultures and added culture supernatant to plates containing *E. coli* OP50 and Orsay virus. We did not observe any potent effect on host susceptibility to infection by Orsay virus from any supernatant (Supp. Fig. 9), although it is difficult to rule out the possibility that the compounds may act at concentrations that are locally higher than the concentrations at which the compounds are present in our experiments.

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

We designed a candidate-based screen using a non-redundant transposon insertion library to gain further insight into the genetic regulation of *P. aeruginosa* mediated reduction of Orsay virus transmission and infection (**Fig. 5A**)[58]. We identified candidate genes to include in our screen from three sources: *gacA*-regulated genes, *rhlR*-regulated genes (but *rhlI*- or *pqsE*-independent), and the set of genes required for full virulence in *C. elegans* previously identified by Feinbaum et al. (**Fig 5A**)[51,59,60]. Of the 211 genes tested, 18 putative hits, including *lasI*, *rhlR*, and *gacA*, were identified with the corresponding *P. aeruginosa* PA14 mutants exhibiting suppression of the attenuation of Orsay virus infection by wild-type *P. aeruginosa* PA14 (**Fig. 6A**, Supp. Table 3). Using the 15 novel candidate genes, we performed an additional set of susceptibility assays which confirmed six of the hits (**Fig. 5B**, Supp. Fig. 10, Supp. Table 3). The identified hits grouped into two clusters based on the strength of their suppression. Transposon insertion in the genes *ptsP*, *prpC*, and *kinB* led to marked suppression of the attenuation of Orsay virus leading to infection of

97%, 89%, and 82% of the population respectively compared to infection in only 4.2% of the population in the presence of wild-type *P. aeruginosa* PA14 (**Fig. 5B**, Supp. Fig. 10). Transposon insertion in three additional genes, *clpA*, *glnK*, and *fabF1* resulted in weaker, but robust suppression of the attenuation of Orsay virus leading to infection of 34%, 28%, and 17% of the population respectively (**Fig 5B**, Supp. Fig. 10). We additionally tested whether transposon insertion into these genes suppressed attenuation of Orsay virus transmission. While we observed a trend towards mutations affecting susceptibility also affecting virus transmission, we observed a high degree of variation in the transmission assay, such that only mutation of *ptsP* led to statistically significant suppression of Orsay virus attenuation (**Fig. 5C**).

We noted that all six of the hits originated from the set of genes required for full *P. aeruginosa* PA14 virulence in *C. elegans*[60]. These hits represented only six out of the 41 tested genes that are known to influence virulence, indicative of some degree of specificity in the consequences of these mutations beyond their general effect on *P. aeruginosa* PA14 virulence[60].

P. lurida MYb11 *gacA* is required for attenuation of Orsay virus transmission

We next sought to determine whether our findings from the interaction of *C. elegans* and Orsay virus in the presence of *P. aeruginosa* could inform us further regarding the mechanisms underlying the attenuation of Orsay virus transmission in the presence of *P. lurida* MYb11. In particular, we identified *gacA, ptsP, prpC, and kinB* orthologs using Orthovenn2 and generated a putative knockout allele of each gene by removing the coding potential for all but 10 amino acids from the N and C termini (**Fig. 6A**)[61]. Following exposure to exogenous Orsay virus, knockout of *gacA* in *P. lurida* MYb11 led to infection of 77% of the population compared to infection of 43% of the population in the presence of wild-type *P. lurida* MYb11 from exogenous Orsay virus (**Fig. 6B**, Supp. Fig. 11A and 11B). These observations were confirmed via FISH, as the *gacA* mutant supported infection of 62% of the population versus 37% infection in the presence of wild-type *P. lurida* MYb11 (**Fig. 6C**). The *gacA* mutation also suppressed the attenuation of Orsay virus transmission, increasing the incidence proportion 2.9-fold compared to wild-type *P. lurida* MYb11 (**Fig. 6D**). These data suggest that *gacA* has a conserved role between *P. aeruginosa* and *P. lurida* MYb11 in the attenuation of Orsay virus transmission and infection of *C. elegans*. On the other hand, knockout of the *P. lurida* MYb11 orthologs of *ptsP*, *prpC*, or *kinB* failed to suppress the attenuation of Orsay virus infection or transmission by *P. lurida* MYb11 (**Fig. 6E**, Supp. Fig. 11C and 11D)[60,62]. One explanation of these results is that these genes play different roles within *P. lurida* MYb11 and *P. aeruginosa* and therefore their mutation did not have the same consequences for Orsay virus attenuation. Alternatively, these results might suggest that *ptsP*, *prpC*, and *kinB* act to attenuate Orsay virus infection via some specific effect on *P. aeruginosa* PA14 virulence but do not influence the interaction of *P. lurida* MYb11 with *C. elegans*.

O. vermis MYb71 closely interacts with the *C. elegans* intestinal brush border

P. lurida MYb11 and *O. vermis* MYb71 can both be isolated from *C. elegans* natural environment[36,39]. We were therefore curious how exposure to both bacteria simultaneously would impact Orsay virus infection rates. When cultures of *O. vermis* MYb71 and *P. lurida* MYb11 were concentrated to 25mg/mL and then mixed, we observed that a 50% mixture of each bacterium (v:v) attenuated infection to the same degree as pure *P. lurida* MYb11 (**Fig. 7A**, Supp. Fig. 11A-B). Moreover, we also noted that mixing *O. vermis* MYb71 with *E. coli* OP50 also lead to infection rates similar to pure *E. coli* OP50 (**Fig. 7A**, Supp. Fig. 11A-B). Previous studies have identified that *Ochrobactrum* is found in large numbers in the *C. elegans* intestine following exposure[36,39]. We investigated how the levels of *Ochrobactrum* in the intestine changed following exposure to pure or mixed lawns of bacteria. When alone, GFP expressing *Ochrobactrum* BH3 was readily observed within the intestine at 4 or 24 h of exposure.

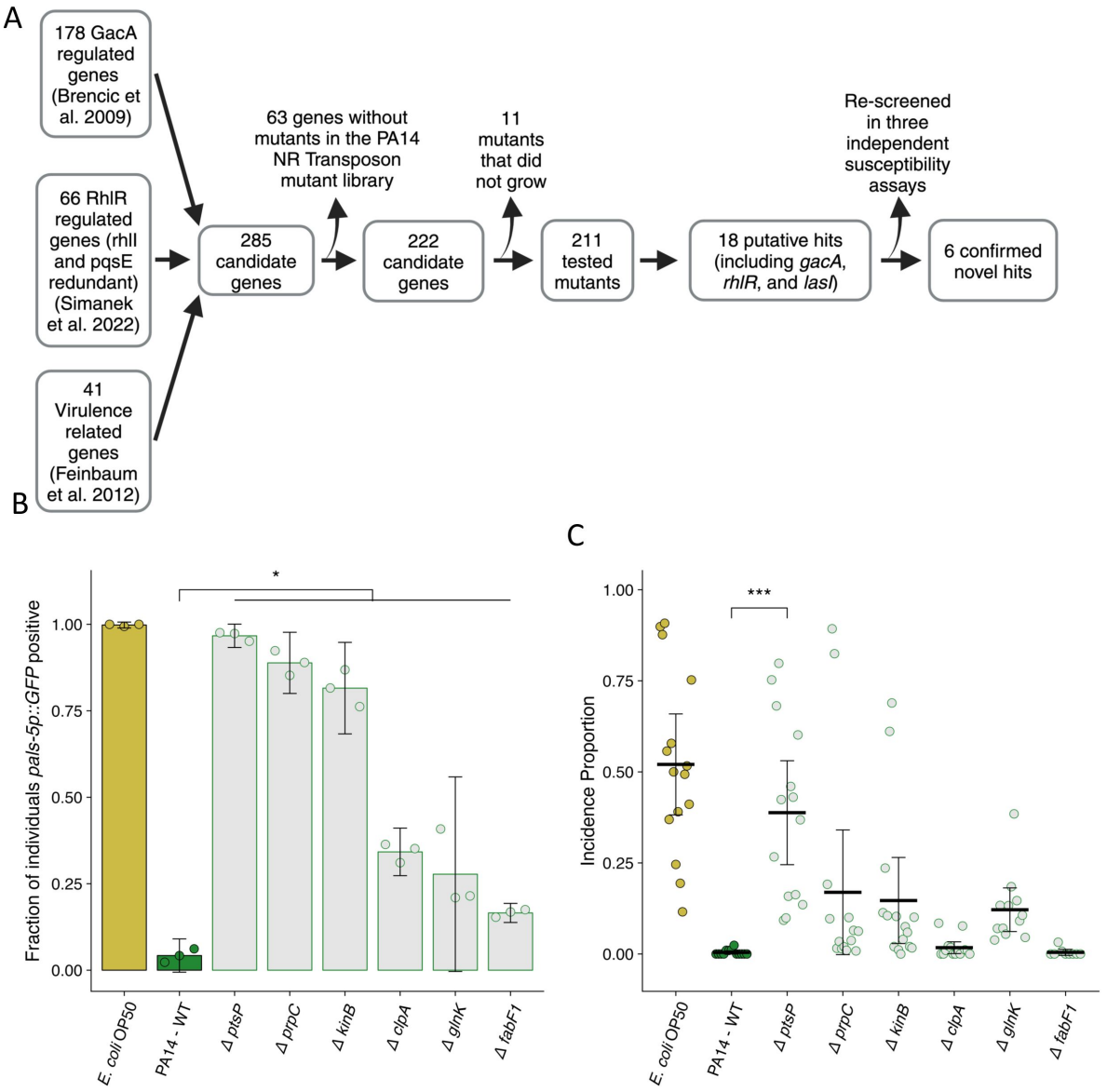

Figure 5

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 *pals-5p::GFP* positive following exposure to 100 a.u. of exogenous Orsay virus. Data are from a single representative experiment. Replicates can be found in Supp. Fig. 10. (n=3779 in total and n > 55 for all dots) (**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 combined and dots represent individual plates. (n=11279 in total and n > 35 for all dots) 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).

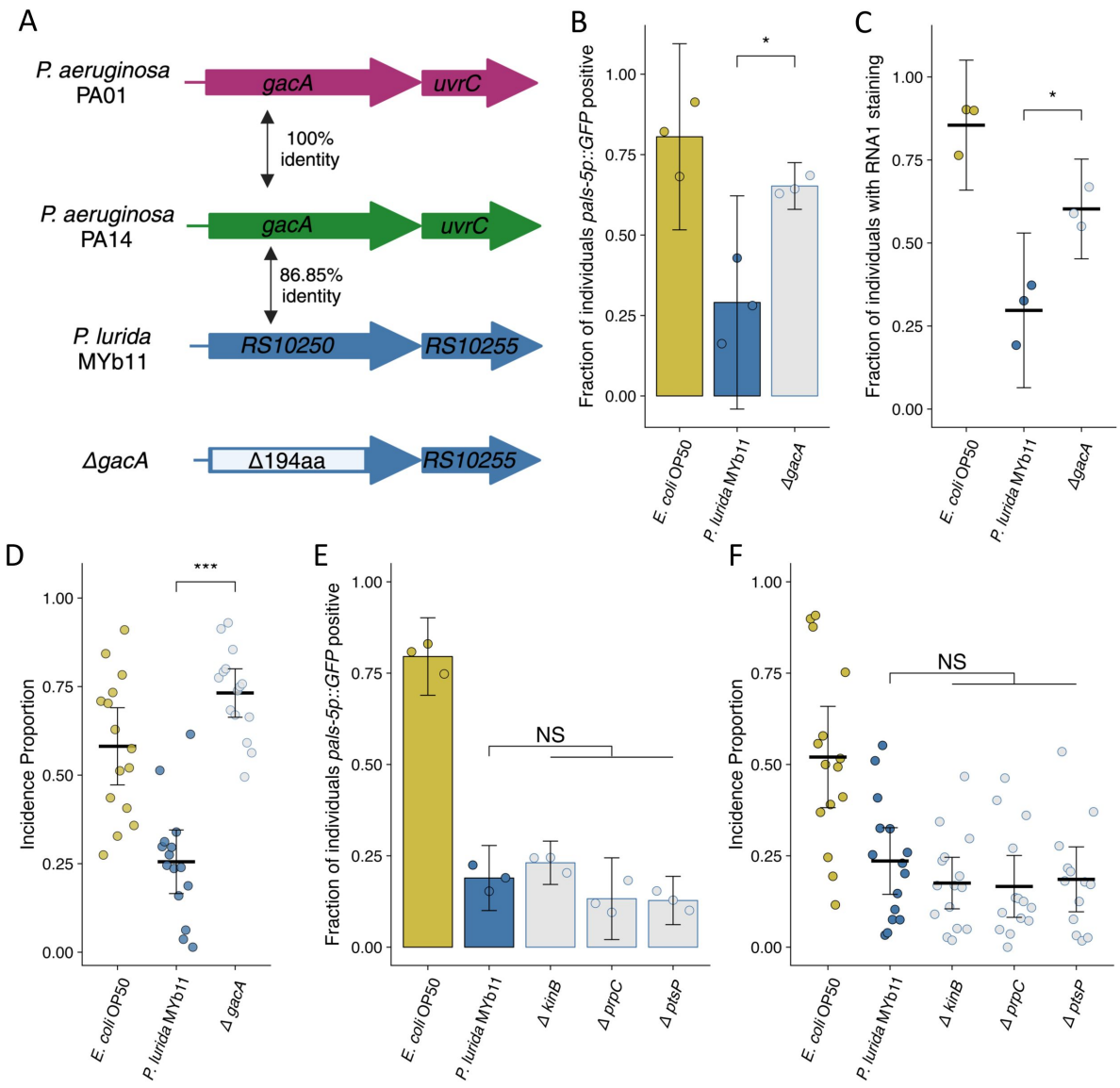

Figure 6

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. Schematic made using Biorender. **(B, E)** The fraction of individuals *pals-5p::GFP* positive following exposure to 10 a.u. exogenous Orsay virus in the presence of wild-type *P. lurida* MYb11 compared to the indicated mutants. Data are from a single representative experiment. Replicates can be found in Supp. Fig. 11. ((B) n=933 in total and n > 46 for all dots, (E) n=1725 in total and n > 78 for all dots). Bars represent mean and dots represent individual plates. **(C)** The fraction of individuals with staining following exposure to 10 a.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 (B) and Supp. Fig 11A and 11B (n=1272 in total and n > 61 for all dots). **(D, F)** Incidence proportion of Orsay virus transmission in the presence of wild-type *P. lurida* MYb11 versus the indicated mutants. Data are from three experiments combined, bars represent mean, dots represent individual plates ((D) n=4534 in total and n > 42 for all dots, (F) n=8185 in total and n > 58 for all dots). For all plots error bars represent 95% confidence interval (C.I.). (B-D) p-values were determined using Student's t-Test. (E-F) p-values determined using one-way ANOVA followed by Dunnett's test (NS non-significant, *p<0.05, **p<0.01, ***p<0.001).
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Interestingly, exposure to a mixed lawn of *Ochrobactrum* BH3 and *P. lurida* MYb11 reduced intestinal accumulation of *Ochrobactrum* BH3 compared to the pure *Ochrobactrum* BH3 treatment (**Fig 7B** and **7C**, Supp Fig. 11C-F). On the other hand, exposure to a mixed lawn of Ochrobactrum BH3 and *E. coli* OP50 reduced accumulation of *Ochrobactrum* BH3 at four hours compared to a pure *Ochrobactrum* BH3 treatment, but this difference was minimal by 24 hours (**Fig 7B** and **7C**, Supp Fig. 11C-F).

These data suggested that the presence of *Ochrobactrum* in the intestine may be important for its ability to promote Orsay virus infection. We therefore fixed *C. elegans* exposed to *O. vermis* MYb71 or *E. coli* OP50 for examination via electron microscopy and imaged cross sections of the intestine. As expected from our fluorescence experiments, *O. vermis* MYb71 was readily observed in the intestinal lumen at 4 or 24 h of exposure (**Fig. 7D** and **7E**). The glycocalyx is the mucus-like layer covering *C. elegans* intestinal cells and that recent work has suggested may play a role in defense against Orsay virus[63,64]. The glycocalyx of animals exposed to *O. vermis* MYb71 was observed with regions of variable thickness and potential instances of *O. vermis* MYb71 deforming the microvilli brush border as opposed to animals exposed to *E. coli* OP50 where the glycocalyx appeared uniform and undisturbed (**Fig. 7D** and **7E**). A recent report observed that *Serratia marcescens* promoted infection of the mosquito *Aedes aegypti* by Dengue, Zika, and Sindbis viruses by secreting a protein, enhancin, that degrades the mucus layer covering epithelial cells[14]. These observations lead us to speculate that *O. vermis* MYb71 mediated disruption of the brush border and glycocalyx promotes Orsay virus infection although this hypothesis has yet to be tested.

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 magnitude in the presence of *O. vermis* MYb71 versus *P. lurida* MYb11 which are found in the natural environment in association with *C. elegans*[36,39]. Moreover, we observe that pathogenic *P. aeruginosa*, which may be found in natural environments, can further attenuate Orsay virus transmission[31]. Our work is also consistent with a recent study by González and Félix that also reported that monoaxenic cultures of other bacteria from the environment of *C. elegans* impact host susceptibility[65].

Our observations that two *Ochrobactrum* species promoted transmission of Orsay virus are intriguing given that other *Ochrobactrum* species have also been linked to viral infections. *Ochrobactrum intermedium* promoted poliovirus infection in mice and enhanced poliovirus stability in vitro[13]. *Ochrobactrum anthropi*, an opportunistic human pathogen, was identified using a random forest analysis as one of the most predictive features differentiating the upper respiratory tract of human patients recovering from influenza infection versus healthy controls[66]. Our observations in the *C. elegans* host raise the speculative possibility that *Ochrobactrum* colonization may have roles in evolutionarily diverse hosts in modulating viral infection. Further, our electron micrographs point to an intriguing hypothesis that *Ochrobactrum* disrupts the brush border region of epithelial cells thereby promoting viral infection. Such a mechanism would be consistent with recent work identifying that *Serratia marcescens* produces the protein enhancin, which promotes viral infection by degrading the mucus layer covering epithelial cells in the mosquito *Aedes aegypti*[14]. Additional work will need to be performed to explicitly test whether such a mechanism is at play in *Ochrobactrum* mediated enhancement of Orsay virus infection and transmission.

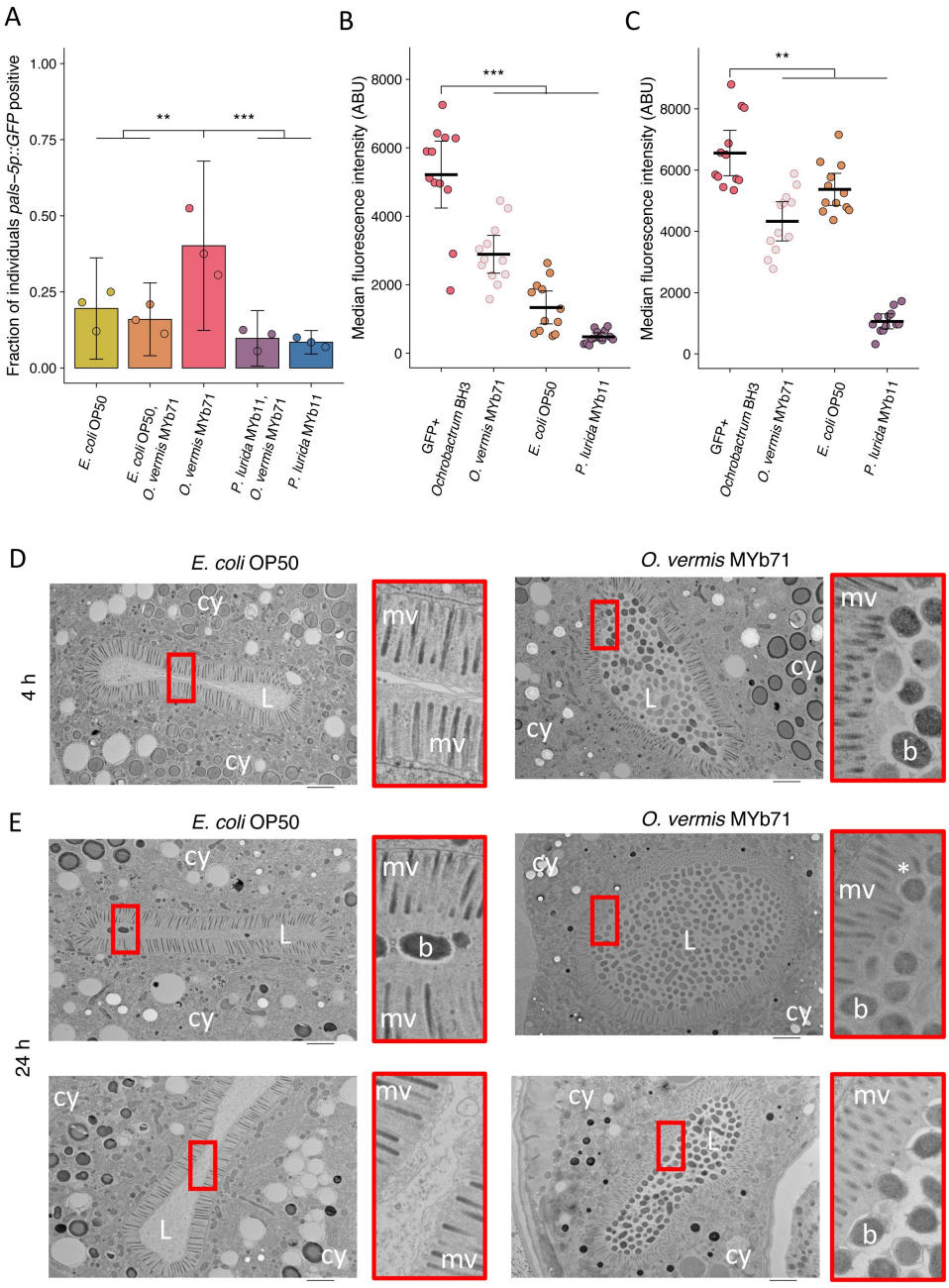

Figure 7

O. vermis MYb71 closely interacts with the brush border of *C. elegans* intestinal cells.

(A) The fraction of individuals *pals-5p::GFP* positive following exposure to 1 a.u. exogenous Orsay virus in the presence of pure lawns of the indicated bacteria or mixed lawns consisting of 50% of the indicated bacteria mixed with 50% *O. vermis* MYb71 (v:v, Methods). Data are from a single representative experiment. Replicates can be found in Supp. Fig. 12. Bars represent mean and dots represent individual plates (n=908 in total and n > 36 for all dots). **(B-C)** The median fluorescence observed in the intestinal lumen of individuals following exposure for (B) 4 h or (C) 24 h to pure GFP expressing *Ochrobactrum* BH3 lawns or mixed lawns consisting of 50% of the indicated bacteria mixed with 50% GFP expressing *Ochrobactrum* BH3 (v:v, Methods). Data are from a single representative experiment. Replicates can be found in Supp. Fig. 12. Bars represent mean, dots represent individual animals. (**D-E**) Electron micrographs of animals exposed to the indicated bacterium for either (D) 4 or (E) 24 h. Scale bar is 2 microns. cy=cytoplasm, L=lumen, mv=microvilli, b=bacterium, and * denotes a bent microvilli. For all plots error bars represent 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).

P. lurida MYb11 and *P. aeruginosa* PA01 and PA14 shared the ability to attenuate Orsay virus infection and transmission. *P. lurida* MYb11 promotes developmental rate and fitness at a cost to overall lifespan[39,46]. In contrast, *P. aeruginosa* rapidly kills *C. elegans* and is detrimental to host fitness[30,44]. Under our experimental conditions exposure to all three shortened lifespan compared to exposure to *E. coli* OP50. Given that *O. vermis* MYb71 shortened lifespan to the same extent as *P. aeruginosa* PA14 but had the opposite effect on host susceptibility to Orsay virus, we do not believe that general virulence by any bacterium is responsible for the observed effects on host susceptibility. Rather, unique virulence processes specific to each bacterium may contribute to the unique effects of each bacterium on host susceptibility to Orsay virus.

We identified that *gacA* regulates the attenuation of Orsay virus by *P. aeruginosa* PA01 and PA14 and *P. lurida* MYb11. In Pseudomonads, *gacA/gacS* regulate processes related to quorum sensing and virulence[67]. Quorum signaling influences additional aspects of *P. aeruginosa* physiology, including swarming behaviors, biofilm formation, secondary metabolism rates, and overall transcription patterns[48]. To gain additional insights into the *P. aeruginosa* PA14 effects on Orsay virus transmission we conducted a candidate-based screen of genes influenced by quorum signaling and genes that regulate virulence towards *C. elegans*[51,59,60]. We identified six additional genes, *ptsP*, *prpC*, *kinB*, *clpA*, *glnK*, and *fabF1*, that when mutated suppressed *P. aeruginosa* PA14 attenuation of Orsay virus infection. Each of these genes, in addition to *gacA*, *rhlR*, and *lasI* has been shown to be required for full *P. aeruginosa* PA14 virulence in *C. elegans*[60]. However, we observed that only these six out of the total 41 virulence-related genes identified in Feinbaum *et al.* influenced Orsay virus infection, arguing further against a role for general virulence in the attenuation of Orsay virus transmission and infection caused by *P. aeruginosa* PA14 and *P. aeruginosa* PA01[60]. Additionally, while mutation of the *ptsP*, *prpC*, and *kinB* orthologs of *P. lurida* MYb11 failed to suppress the attenuation of Orsay virus, it is unclear whether these genes influence the effect of *P. lurida* MYb11 on host lifespan or even whether *P. lurida* MYb11 pathogenicity mediated by other factors leads to attenuation of Orsay virus infection.

C. elegans is unlikely to associate with a single bacterium in its natural environment. However, *C. elegans* shows clear behavioral preferences for grazing on certain bacteria from its environment and may eat monoxenic lawns in the wild[68]. Our data demonstrate that individual bacteria can have a profound impact on Orsay virus transmission rates in a species-specific manner. Additionally, the tractability of the *C. elegans*-Orsay virus experimental system allowed us to identify molecular determinants of viral transmission and will be useful for identifying additional biotic factors that influence viral transmission. Our work builds on the expanding body of knowledge showing that the microbiota can influence the interactions between viruses and their animal hosts.

Materials and methods

C. elegans strains and growth conditions

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

Bacterial strains and growth conditions

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

Orsay virus isolation and batch testing

Orsay virus was isolated from infected WUM31(*rde-1(ne219);jyIs9[pals-5p::gfp;myo-2p::mCherry]*) individuals. Plates with gravid WUM31 were bleached to obtain eggs. Eggs were hatched overnight in M9 solution rotating at 20°C and the L1 larvae were arrested to synchronize the population. L1 larvae were combined with 100 µL of 6x concentrated *E. coli* OP50 liquid culture and 50 µL of Orsay virus filtrate, plated on SKA plates, and once dried, placed at 20°C for 48 h. Four GFP positive individuals were then transferred to a new 6cm NGM plate with *E. coli* OP50 and maintained until just starved. Twenty such 6cm plates were washed with 10 mL of M9 and the resulting suspension was Dounce homogenized. Alternatively, two 3.5 cm SKA plates containing infected WUM31 animals were allowed to starve and then equally chunked onto eight 10 cm NGM plates with *E coli* OP50. Once these plates were just starved, the plates were washed with 10 mL of M9 and homogenized as above. The homogenized suspension was then centrifuged at 13,200xg for five min. The supernatant was passed through a 0.22 µm filter and aliquoted. Each batch of virus was tested for potency and an ID_{50} calculated for ZD2611 populations in the presence of *E. coli* OP50 (Supp. Fig. 2). On average, 3.6 µL of Orsay virus stock prepared as indicated in the Methods was required to infect 50% of a population of ZD2611 animals in the presence of *E. coli* OP50 after 24 hours (Supp. Fig. 2). We observed variability in the measured ID_{50} even when using the same batch of Orsay virus (e.g. **Fig. 1D** vs Supp. Fig. 3A vs. Supp. Fig. 3B). We therefore chose to control all our experiments internally rather than attempt to normalize between Orsay virus batches and we report doses used in arbitrary units (a.u.), however 1 a.u. corresponds to 1 µL of Orsay virus filtrate.

Preparation of Uninfected Individuals for transmission and susceptibility assays

Prior to all assays, plates with fecund ZD2611(*glp-4(bn-2);jyIs8[pals-5p::gfp;myo-2p::mCherry]*) were bleached to obtain eggs. Eggs were hatched overnight in M9 solution rotating at 20°C and the L1 larvae were arrested to synchronize the population. ZD2611 L1s were dropped onto plates containing *E. coli* OP50 and placed at 20°C for 64-72 h. At this temperature reproduction is delayed, but not eliminated, and no L1s are observed during transmission or susceptibility assays. The resulting young adults were washed off the plates in M9 and centrifuged at 1000xg for one minute. After removing the supernatant, the young adults were once again washed in M9, then centrifuged at 1000 x g for one minute. Young adults were directly dropped onto prepared plates described below.

Preparation of Bacteria for transmission and susceptibility assays

Unless otherwise indicated, bacteria were cultured overnight and added to cover the assay plates. Plates containing *E. coli* OP50 or *P. aeruginosa* strains were placed at 37°C for 24 h. Plates containing *O. vermis* MYb71 or *P. lurida* MYb11 strains were placed at 25°C for 24 h. All plates were then moved to room temperature for an additional 24 h before the addition of virus.

For the natural isolate transmission screen and early transmission assay cultures were grown to late stationary phase. Cultures were then spun down for six minutes at 4000xg and reconstituted to 25 mg/mL in their own supernatant. 150 µL of this suspension was combined with 50 µL of a

mix of M9 for transmission assays or 50 µL of a combination of Orsay virus filtrate and M9 for the early transmission assay. This mixture was combined with young adult ZD2611 individuals and directly added to cover assay plates before drying.

Transmission assays

To obtain infected spreader animals, fecund ZD2610 (*glp-4(bn-20); rde-1(ne219); jyIs8[pals-5p::gfp;myo-2p::mCherryJ*) were bleached to obtain eggs. Eggs were hatched overnight in M9 solution rotating at 20°C and the L1 larvae were arrested to synchronize the population. Arrested ZD2610 L1s were dropped onto NGM plates containing *E. coli* OP50 and placed at 20°C for 48 h. Animals were then washed off the plates in M9 and centrifuged at 1000xg for one min. After removing the supernatant, the young adults were washed with M9 again then centrifuged at 1000xg for one min. Supernatant was once again removed, and individuals were immediately mixed with 100 µL of 6x overnight *E. coli* OP50 culture and 50 µL of Orsay virus for a minimum of 18-20 h. *pals-5p::GFP*-positive ZD2610 young adults were identified and picked onto transfer plates containing the appropriate bacteria prepared as indicated above for 4-6 h. After this period, five spreaders were transferred to assay plates containing the same bacteria and approximately 100 uninfected ZD2611 individuals to start the assay. Transmission assays were scored 24 h after adding spreader individuals.

For the natural isolate transmission screen, arrested ZD2610 L1s were combined with 100 µL of 6x concentrated *E. coli* OP50 and 50 µL of Orsay virus filtrate and once dried, placed at 20°C for 64-72 h. On the day of the assay, *pals-5p::GFP*-positive ZD2610 young adults were identified and picked onto transfer plates containing the appropriate bacteria prepared as indicated above for 4-6 h. After this period, five spreaders were transferred to assay plates containing the same bacteria and approximately 100 uninfected ZD2611 individuals to start the assay.

Incidence proportion

Incidence proportion was calculated by dividing the number of newly infected animals by the total non-spreader population per plate. Individual plates for which the total number of *pals-5p::GFP* positive individuals after 24 h was less than the initial number of spreaders placed on the plate were excluded to eliminate potential differences in incidence proportion caused by unequal numbers of infected spreader animals. For **Fig. 1C** a single plate was censored from *P. lurida* MYb11, for **Fig. 2A** six plates were censored from *P. aeruginosa* PA14, and for **Fig. 4E** 12 plates were censored: seven from *P. aeruginosa* PA14, three from *P. aeruginosa* PA01, one from *P. aeruginosa* PA14Δ*gacA*, and one from *P. aeruginosa* PA14Δ*lasI*.

Susceptibility assays

Unless otherwise indicated, Orsay virus filtrate at the indicated doses was diluted in filtered M9 solution. 200 µL total was applied to each assay plate and swirled to cover the entire bacterial lawn. Plates were then dried before the addition of approximately 100 uninfected ZD2611 individuals prepared as indicated above. Assays were scored 24 h later using a Nikon SMZ18 stereo fluorescent microscope and the fraction of *pals-5p::GFP*-positive individuals was calculated.

Early Transmission Assay

For early transmission assay, young ZD2611 adults were exposed to 0 a.u. or 5 a.u. exogenous Orsay virus in the presence of *O. vermis* MYb71. 8 hours 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 hours after the transfer.

Supernatant Transfer Assay

Assay plates were prepared with *E. coli* OP50 for susceptibility assays as indicated above. Bacteria were inoculated into 6 mL of LB and allowed to grow for 48 h. Cultures were then spun at 4,000xg for 6 minutes and the spent media supernatant was passed through a 0.22 µm filter. 200 µL of the filtered supernatant was added to the *E. coli* OP50 assay plates just prior to the addition of Orsay virus and ZD2611 young adults.

Fluorescence *in situ* hybridization

Previously published probes were obtained to target the RNA1 segment of Orsay virus[25]. Animals were infected according to the methods described above for susceptibility assays. Animals were processed according to the Stellaris RNA FISH Protocol for *C. elegans* (LGC Biosearch Technologies) with minor modifications. Briefly, young adults were washed off the plate and rinsed twice in M9. Animals were then fixed for 30 min rotating in a microcentrifuge tube at 20°C. After washing twice with 1 mL of phosphate buffered saline animals were permeabilized in 70% of ethanol and stored at 4°C for 1-7 d. Animals were washed with Wash Buffer A before addition of 100 µL of hybridization buffer containing 3 µL of RNA1 probe mix. Probe was hybridized overnight at 46°C. Animals were then washed with Wash Buffer A alone once, and then again with Wash Buffer A containing 5 ng/mL DAPI. Lastly 100 µL of Wash Buffer B was added before mounting animals on slides with 25 µL of Vectashield Mounting Medium. The fraction of individuals with RNA1 staining was then quantified using a Nikon SMZ18 stereofluorescent microscope or a Zeiss AxioImager Z1 compound fluorescent microscope.

Viral Stability

Assay plates with each bacterium were prepared as described above. 50 a.u. Orsay virus was added to each plate as in a susceptibility assay however no *C. elegans* were added. Plates were washed with 1 mL of ddH_20 after 30 minutes or 24 h. A spike-in control was also included where 50 a.u. of Orsay virus was directly added to 1ml H_20 and processed the same as all other samples. The resulting mixture was spun at 15000 rpm (max speed) at 4°C for 10 min. 1 µl of the supernatant was used to make cDNA (Promega GoScript reverse transcriptase using random primers (A2801)). Orsay virus RNA1 level was quantified by qPCR using 1 µl of 1/5 diluted cDNA (GoTaq Promega A6001) and run on QuantStudio 3 Real Time PCR system (RNA1 qPCR primers GW194 and GW195)[23].

Viral Shedding

Infected ZD2610 animals were prepared as indicated above with infection occurring at the L4 stage. 24 h later as young adults, 20 *pals-5p::GFP* positive animals were transferred to assay plates prepared with bacteria as indicated above for a transmission assay. These animals were allowed to shed virus onto the plate for 24 h at 20°C. Spreaders were then removed and the assay plates were washed with 1 mL of M9. The resulting mixture was spun at 15000 rpm (max speed) at 4°C for 10 min. 1 µl of the supernatant was used to make cDNA (Promega GoScript reverse transcriptase using random primers (A2801)). Orsay Virus RNA1 level was quantified by qPCR using 1 µl of 1/5 diluted cDNA (GoTaq Promega A6001) and run on QuantStudio 3 Real Time PCR system. (RNA1 qPCR primers GW194 and GW195)[23]

RNA Extraction

Animals were washed 5 times in M9 and collected in TRIzol reagent (Invitrogen) and stored at –80°C before extraction. RNA extraction was performed using Direct-zol RNA microprep kits (Zymo Research) following the manufacturer's instructions.

qPCR

cDNA was made using 1000 ng of RNA as template (Promega GoScript Reverse Transcriptase using Random Primers). cDNA was diluted at 1/80 and qPCR were performed using 1 µl of diluted cDNA (GoTaq Promega) and run on QuantStudio 3 Real Time PCR system. RNA1 levels were then quantified via qPCR using previously published primers[23]. Orsay virus RNA levels were normalized to the internal control host gene snb-1 (primers GCTCAGGTTGATGAAGTCGTC and GGTGGCCGCAGATTTCTC).

Plasmid-based replication experiments

Adult animals carrying a transgene containing the wild-type RNA1 or RNA1D601A Orsay virus genome segment under the control of a heat-inducible promoter were placed on the indicated bacteria for 4 h before heat-shock at 33°C for 2 h[47]. Animals then recovered at 20°C for 20 h before harvesting for RNA extraction and qPCR as detailed above. RNA1 levels were normalized to the values obtained from animals bearing the wild-type RNA1 and exposed to *E. coli* OP50.

Orsay virus replication in the presence of *P. lurida* MYb11 and *P. aeruginosa*

Adult ERT54 (*jyIs8[pals-5p::gfp;myo-2p::mCherryJ*) or WUM31 (*rde-1(ne219); jyIs8[pals-5p::gfp;myo-2p::mCherry)*] were exposed to exogenous Orsay virus in the presence of *P. lurida* MYb11, *P. aeruginosa* PA01, or *P. aeruginosa* PA14 prepared as in a susceptibility assay. After 2 h or 24 h, animals were harvested for RNA extraction and qPCR as detailed above. RNA1 levels were quantified via qPCR. Within each genotype, the data for each experiment were normalized to the 2 h timepoint.

P. lurida MYb11 mutant construction

A protocol developed for allelic exchange in *P. aeruginosa* was modified for use in *P. lurida* MYb11[69]. Briefly, homology arms flanking the region to be deleted were obtained using polymerase chain reaction (PCR) and cloned into the pExG2-KanR suicide vector using Hi-Fi Assembly (New England Biolabs)[70]. DH5α *E. coli* were transformed using a standard heat shock protocol. Successful transformants were selected for on LB+Kanamycin (50 µg/mL) plates and colony PCR was performed to check for proper insert size in the transformants. *E. coli* bearing the desired plasmid were grown overnight in LB+Kanamycin (50 µg/mL). Plasmids were then obtained using a Qiagen MiniPrep Kit (Qiagen). Plasmids were assessed for the desired sequence by Sanger sequencing and transformed into *P. lurida* MYb11 using the following electroporation procedure. *P. lurida* MYb11 was grown overnight then placed on ice for 30 min. The culture was spun down at 4°C and washed twice with ice-cold water. After reconstitution in 100 µL of ice-cold water the suspension was transferred to a pre-chilled cuvette and transformed at 630 kV using an Eporator (Eppendorf). 900 µL of LB was added, and the suspension was transferred to a microcentrifuge tube and incubated at 27°C for 2 h with shaking. The suspension was then plated on LB+Kanamycin (50 µg/mL) plates and grown for 48 h at 25 °C. Colonies were picked and grown overnight in LB. Cultures were streaked onto sucrose plates (15 g agar, 10 g tryptone, 5 g yeast, 60 g sucrose per 1 L water) to perform sucrose-based counter selection[69]. Colonies that survived were genotyped for the expected deletion.

Lifespan Analysis

Assay plates, bacteria, and ZD2611 animals were prepared as indicated above, however plates were prepared with the addition of 50 µg of 5-fluorodeoxyuridine (FUdR) to eliminate the need to transfer animals during the assay by preventing reproduction. 30 young adult ZD2611 animals were added to each assay plate and assessed for survival every 24 h. Three replicate plates were prepared per experiment and three experiments were performed in total.

Pumping Rate

Assay plates, bacteria, and ZD2611 animals were prepared as indicated above. 20 young adult ZD2611 animals were transferred to each assay plate. At 6 h and 24 h, animals were recorded with slow-motion video capture (1/4x) while feeding. Cycles of pharyngeal pumping were counted and used to calculate the number of pharyngeal pumps per minute.

Mixing Susceptibility Assays and Colonization Assays

Bacteria were grown in LB for 48 h before being spun down at 4,000xg for 6 min. The resulting cell pellets were weighed and reconstituted to 25 mg/mL in their own culture supernatant. Concentrated bacteria were then combined with filtered M9 for colonization experiments or filtered M9 plus 1 µL of Orsay filtrate for mixing susceptibility assays. For mixing susceptibility assays young adult ZD2611 individuals prepared as above were added to the mixture before addition to plates such that the bacterial lawn covered the entire plate. For colonization assays N2 animals were prepared in the same manner to adulthood. Mixing susceptibility assays were scored as a susceptibility assay as indicated above. Colonization assays were scored at 4 h or 24 h. Briefly, animals were picked off of the plate and anaesthetized in sodium azide before mounting on a microscope slide. Fluorescent images of the region of the intestinal lumen behind the pharynx were imaged using a Zeiss AxioImager Z.1 compound microscope. Images were then imported into FIJI for analysis[71,72]. An irregular polygon tracing the region behind the pharynx was drawn and the median fluorescence intensity measured.

Electron Microscopy

Animals were prepared for electron microscopy as indicated above for susceptibility assays. Animals were washed off their plates in M9 and allowed to pellet by gravity. The supernatant was removed and animals were anesthetized with sodium azide. Animals were then pipetted into a type A 6mm Cu/Au carrier (Leica) and frozen in a high-pressure freezer (EM ICE, Leica). This was followed by the following freeze-substitution protocol: (EM AFS2, Leica)

Freeze substitution protocol

Cocktail 1: 2% osmium tetroxide, 0.1% uranyl acetate in anhydrous acetone

Program

Cocktail	Temperature (start)	Temperature (end)	Time
Cocktail 1	-90 °C	-90 °C	60 h
Cocktail 1	-90 °C	-20 °C	11 h
Cocktail 1	-20 °C	-20 °C	10 h
Cocktail 1	-20 °C	0 °C	12 h
Acetone wash (4x)	0 °C	22 °C	2 h

After the washes in acetone, samples were incubated in propylene oxide for 30 min, then slowly infiltrated in TAAB Epon (TAAB Laboratories Equipment Ltd, *https://taab.co.uk*) as follows:

3:1 Propylene Oxide:TAAB Epon – 3hrs at RT

1:1 Propylene Oxide:TAAB Epon – ON at 4C

1:3 Propylene Oxide:TAAB Epon – 4 hrs at RT

100% TAAB Epon – 2 hrs at RT

Samples were embedded in fresh TAAB Epon and polymerized at 60 degrees C for 48 hrs.

Ultrathin sections (about 80nm) were cut on a Reichert Ultracut-S microtome, picked up on to formvar/carbon coated copper grids, sections were stained with 0.2% lead citrate. The sections were examined in a JEOL 1200EX Transmission electron microscope and images were recorded with an AMT 2k CCD camera.

Data visualization and statistics

All experiments were performed three times. For transmission, qPCR, FISH-based susceptibility assays, and lifespan assays data from each experiment are combined. For susceptibility assays and colonization assays a single representative experiment is shown and replicates are shown in the supplementary material. Data were analyzed in R Studio[73]. When comparing all the means of more than two groups p-values were calculated using one-way ANOVA followed by the Tukey HSD test. When comparing multiple experimental groups to a control group p-values were calculated using one-way ANOVA followed by Dunnett's test. P-values for assays comparing only two groups were calculated using Student's t-test or Welch's t-test as indicated. *E. coli* OP50 is included in all experiments as a reference but was not included for statistical comparison unless explicitly noted. Susceptibility assay curves were modeled using the drc[74] package in R. A two-parameter log-logistic function was used to model the curve and ID_{50} values were calculated using the ED function. Kaplan-Meier survival curves were made using the survival[75] package and curves were compared using the log-rank test within the survdiff function. Plots were made using the gdata[76], scales[77], drc[74], Rmisc[78], multcomp[79], survival[75], ggplot2[80], ggsignif[81], and cowplot[82] packages.

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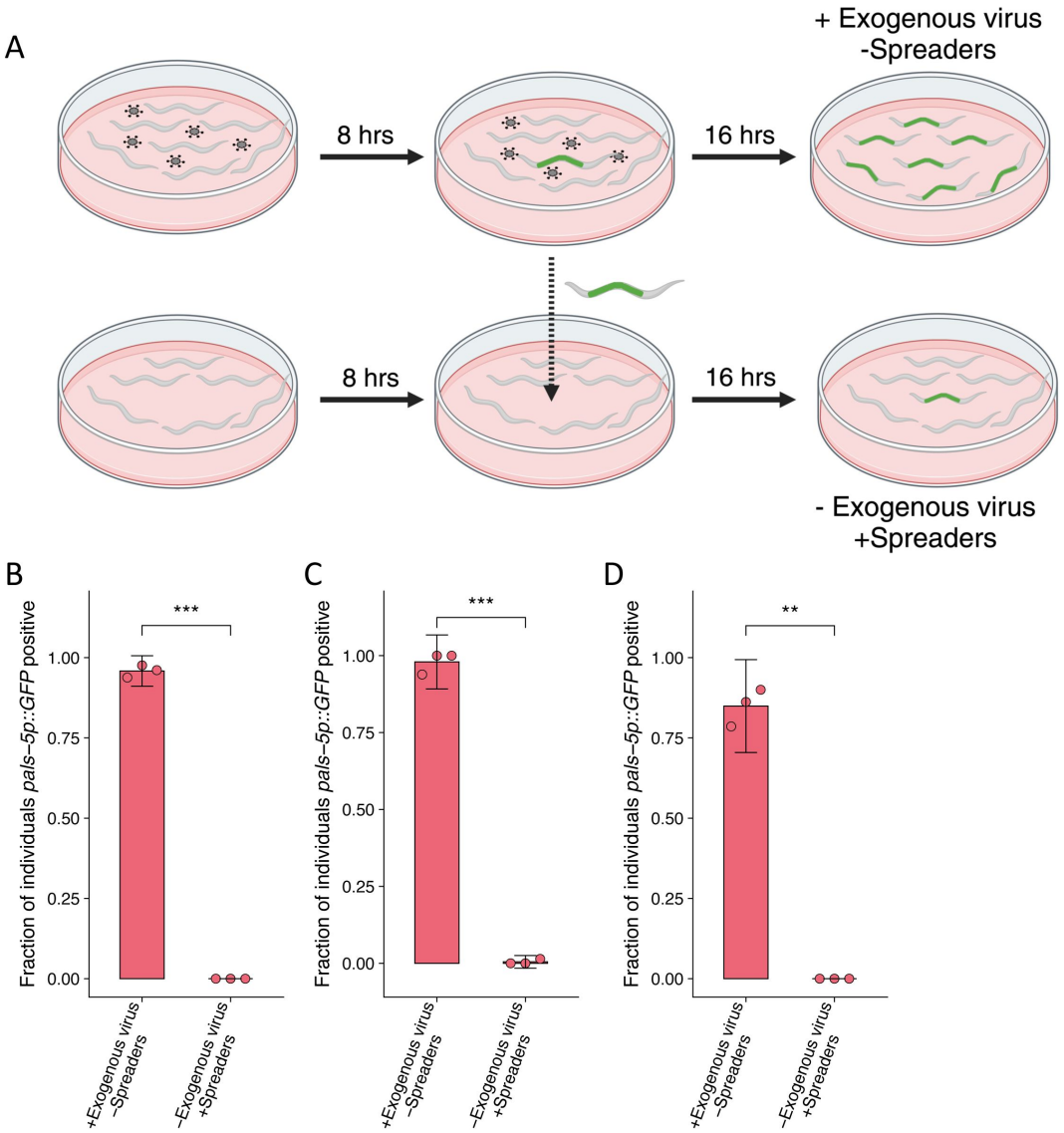
Supplementary Information



Supplementary Figure 1

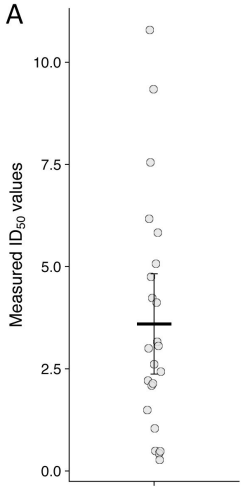
Transmission does not occur from individuals infected during the course of a transmission or susceptibility assay.

(A-D) 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 for each plot are from a single representative experiment, bars represent mean, dots represent individual plates ((B) n = 669 in total and n > 83 for all dots, (C) n = 377 in total and n > 52 for all dots, (D) n=273 in total and n > 33 for all dots). 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

The range of all ID_{50} values calculated for each batch of Orsay virus.

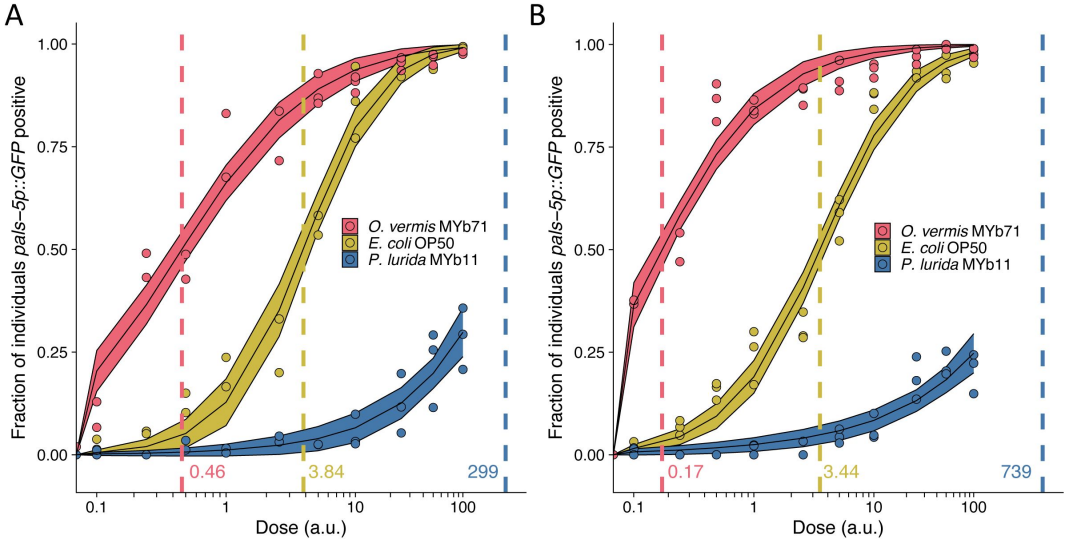
(A) Dose response curves as shown in **Figure 1D** and Supp. Fig. 3 were measured for ZD2611(*jyIs8[pals-5p::GFP; myo-2p::mCherry];glp-4(bn2ts)*) animals exposed to *E. coli* OP50 for each batch of Orsay virus used. The dose of virus required to infect 50% of the population, the ID_{50}, was calculated and plotted. Bar represents the mean and individual dots represent the ID_{50} from a separate dose response curve. Error bars are the 95% confidence interval (C.I.).



Supplementary Figure 3

Replicate dose response curves shown in Figure 1D.

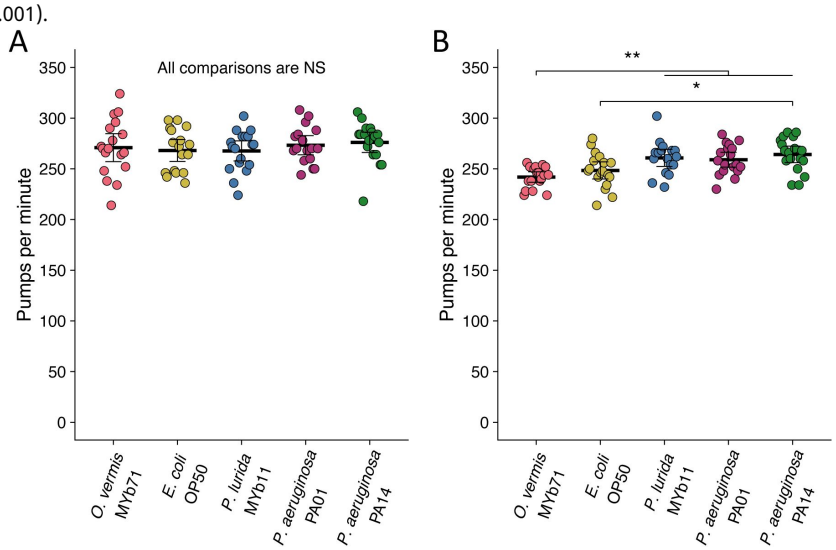
(A-B) Dose response curves of *C. elegans* to exogenous Orsay virus. The dashed line indicates the calculated dose at which 50% of the population was infected, the ID_{50}, the exact value which is given above the x-axis for each bacterium. The solid curves represent the 95% confidence interval of the modeled log-logistic function while in the presence of each bacterium. Data are from a single representative experiment, Dots represent individual plates((A) n = 11350 in total and n > 38 for all dots, (B) n = 7753 in total and n > 24 for all dots). ((a.u.), arbitrary units).



Supplementary Figure 4

Pharyngeal pumping rates are unaltered at 6 h and change only sightly by 24 h after exposure to novel bacteria.

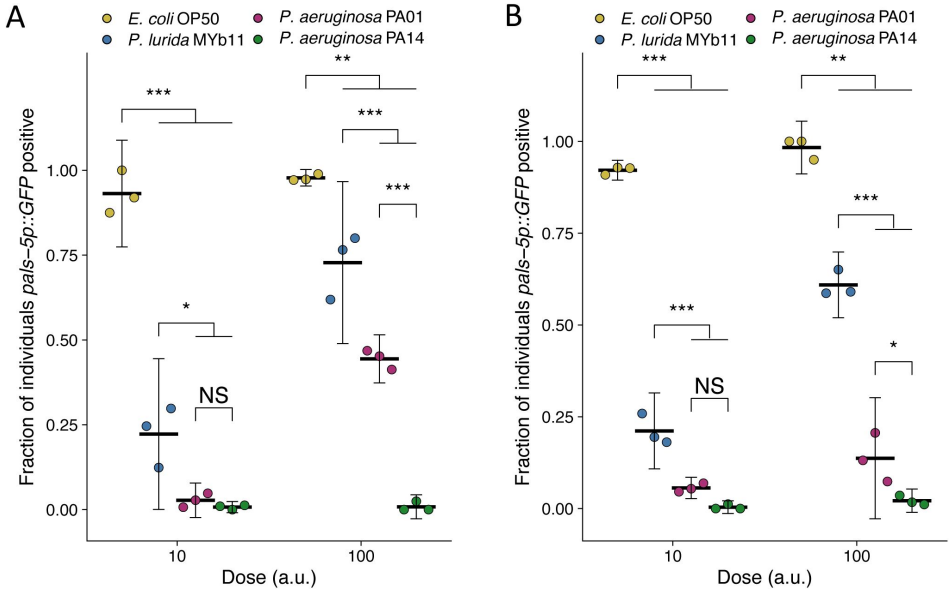
(A-B) Pharyngeal pumping rate of ZD2611 animals exposed to the indicated bacteria for **(A)** 6 or **(B)** 24 h. Pumping rate was assessed via slow-motion video capture of animals feeding (Methods). Data are from three separate experiments and each dot represents a single animal. The black bar is the 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).

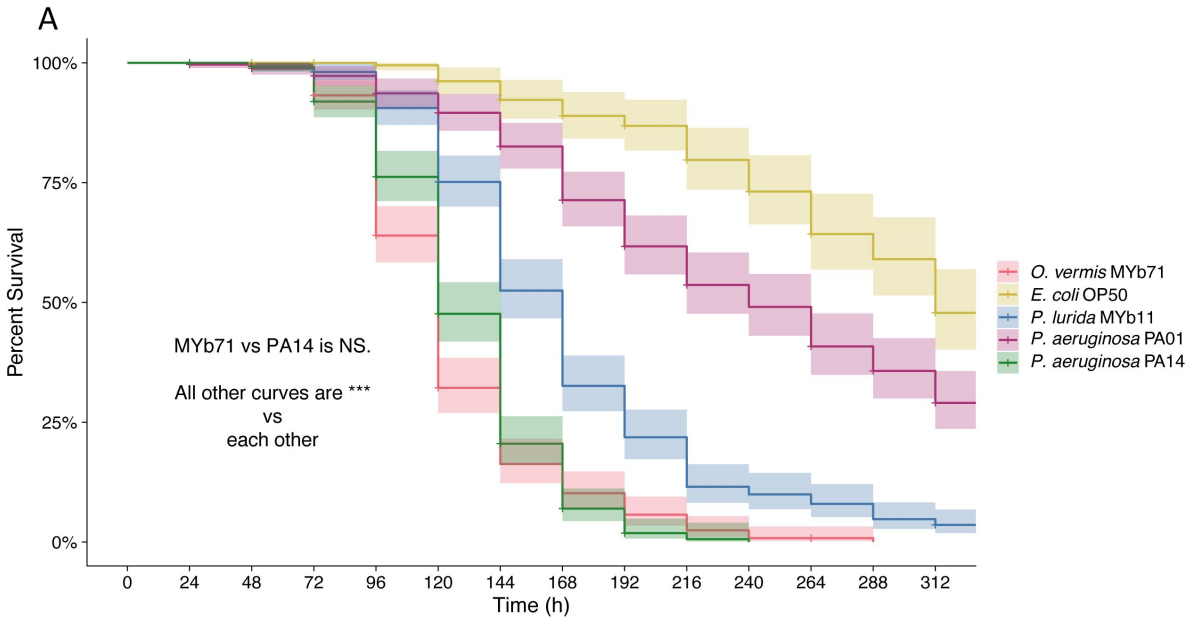


Supplementary Figure 5

Replicate susceptibility assays shown in Figure 2B.

(A-B) The fraction of individuals *pasl-5p::GFP* positive following exposure to two doses of exogenous Orsay virus. Data are from a single representative experiment and dots represent individual plates ((A) n = 2729 total and n > 60 for all dots, ((B) n = 2051 total and n > 48 for all dots). 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).





Supplementary Figure 6

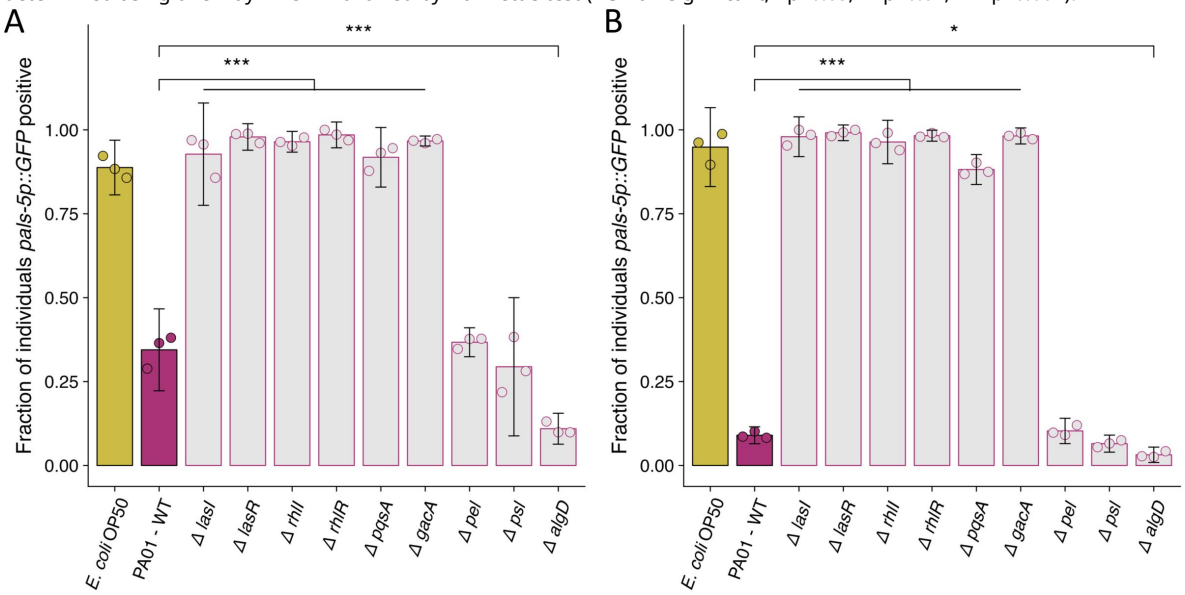
O. vermis MYb71, *P. aeruginosa* PA14, *P. lurida* MYb11, and *P. aeruginosa* PA01 reduce lifespan compared to *E. coli* OP50.

(**A**) Survival of ZD2611 animals following exposure to full lawns of the indicated bacteria on fluorodeoxyuridine (FUdR) containing plates at 20°C. Hashes indicate censored animals. Lines represent the Kaplan-Meier curve, while shaded area represents the 95% confidence interval. Curve and confidence interval were calculated from three separate experiments with three replicate plates for each bacteria with approximately 30 individuals per plate. Statistics were calculated with the log-rank test implemented in the survdiff function in the survival package in R followed by the Bonferroni correction. (***p<0.001).

Supplementary Figure 7

Replicate susceptibility assays shown in Figure 4B.

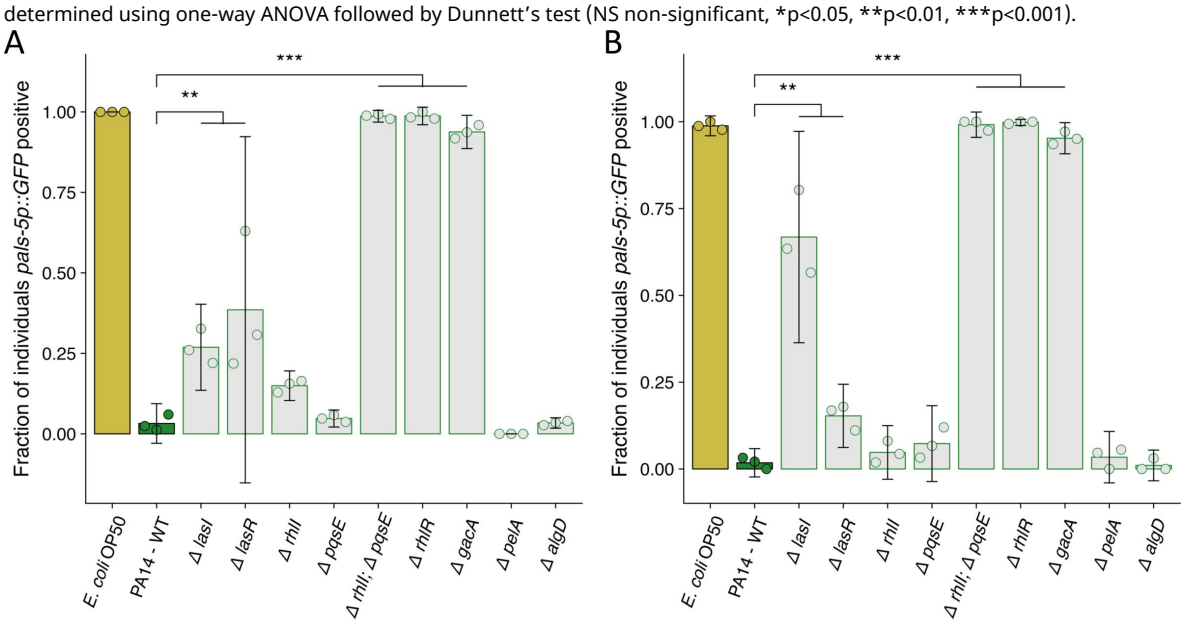
(A-B) The fraction of individuals *pals-5p::GFP* positive following exposure to 10 a.u. of exogenous Orsay virus. Data are from a single representative experiment and dots represent individual plates ((A) n = 2627 in total and n > 43 for all dots, (B) n = 4068 in total and n > 75 for all dots). The black 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).

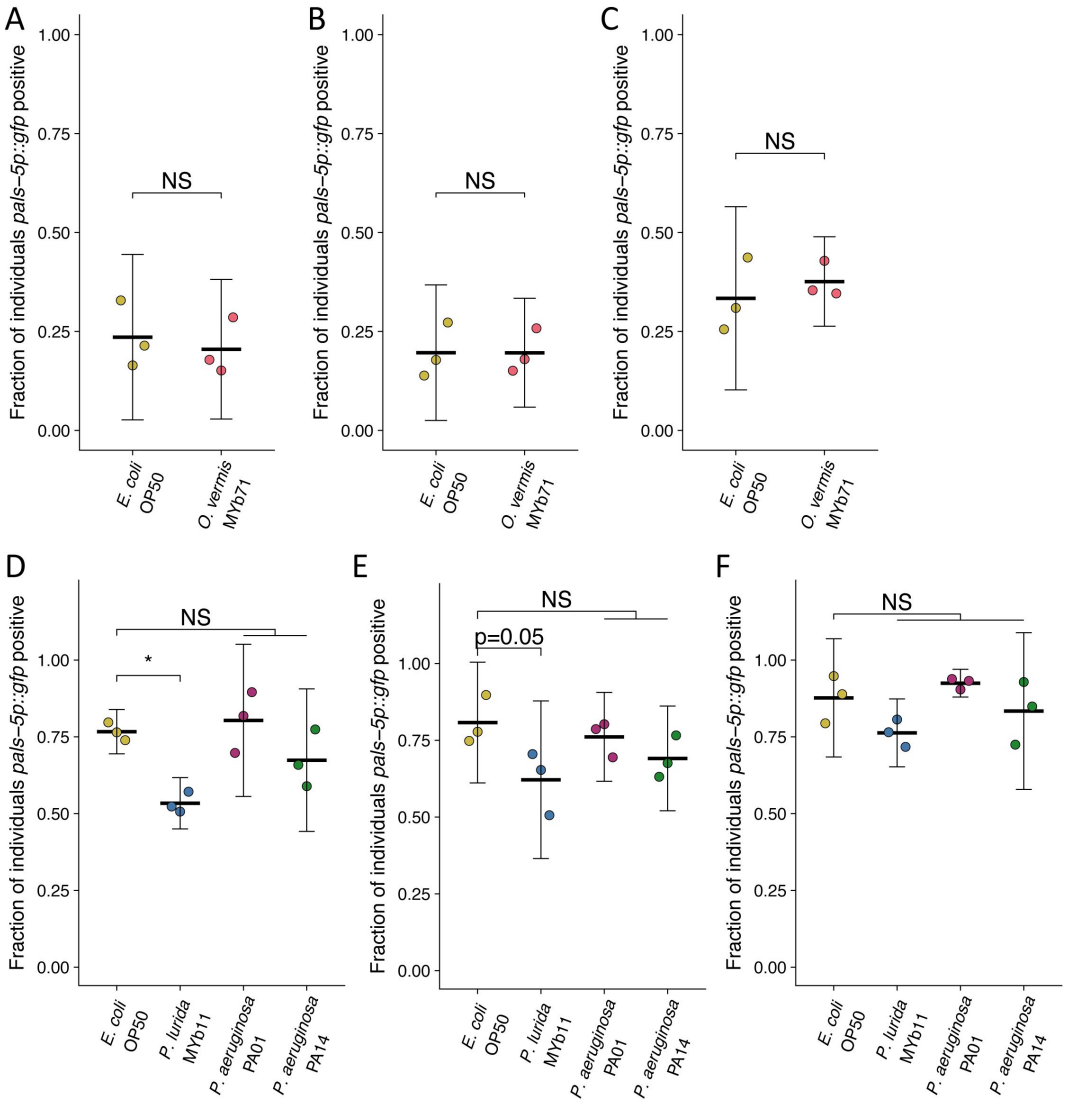


Supplementary Figure 8

Replicate susceptibility assays shown in Figure 4C.

(A-B) The fraction of individuals *pals-5p::GFP* positive following exposure to 100 a.u. of exogenous Orsay virus. Data are from a single representative experiment and dots represent individual plates ((A) n = 3672 in total and n > 37 for all dots, (B) n = 2728 in total and n > 23 for all dots). 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).

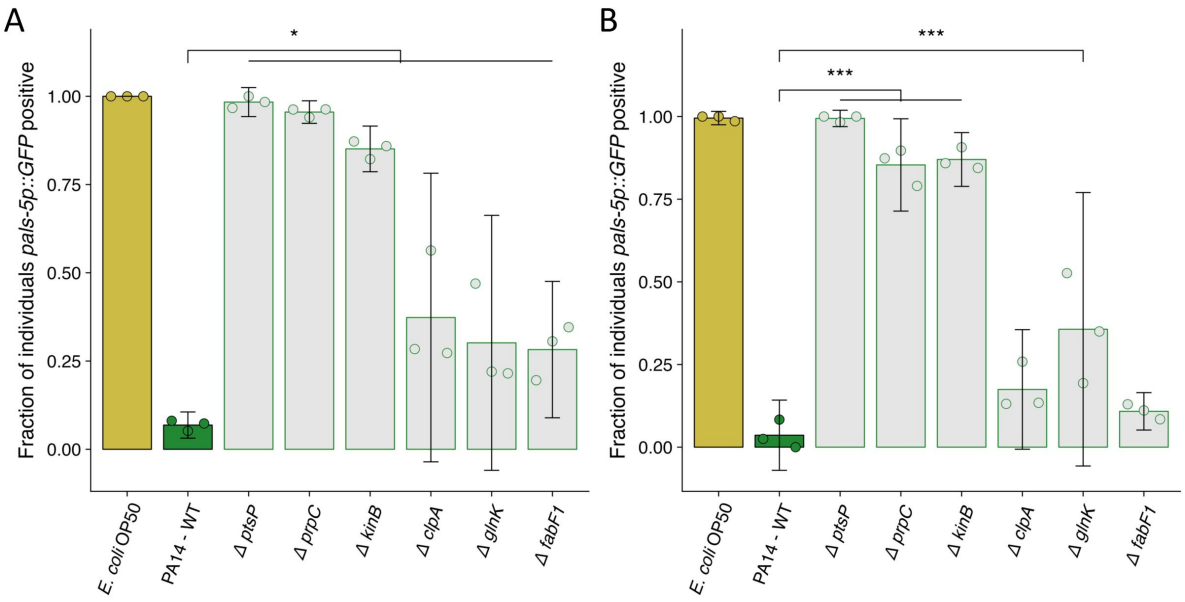




Supplementary Figure 9

Supernatant from stationary phase cultures of different bacteria does not reliably attenuate or enhance Orsay virus infection in the presence of *E. coli* OP50.

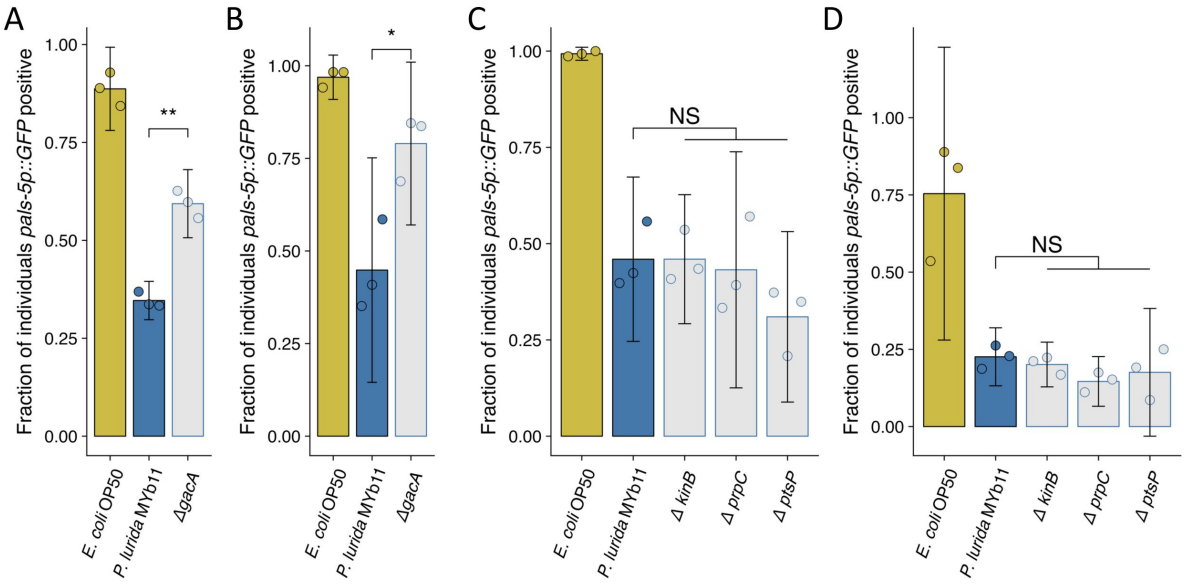
(A-F) The fraction of individuals GFP positive following exposure to (A-C) 0.5 a.u. or (D-F) 5 a.u. of exogenous Orsay virus in the presence of *E. coli* OP50 saturated stationary phase supernatant from the indicated bacteria. Data from each plot are from a single experiment. The black bar is the mean, error bars are the 95% confidence interval (C.I.), and dots represent individual plates ((A) n = 384 in total and n > 35 for all dots, (B) n = 525 in total and n > 55 for all dots, (C) n = 881 in total and n > 103 for all dots, (D) n = 843 in total and n > 46 for all dots, (E) n = 1040 in total and n > 39 for all dots, (F) n = 1603 in total and n > 86 for all dots). (A-C) p-values determined using Student's t-test. (D-E) p-values determined using a one-way ANOVA followed by Dunnett's test (NS non-significant, *p<0.05, **p<0.01, ***p<0.001).



Supplementary Figure 10

Replicate susceptibility assays shown in Figure 5B.

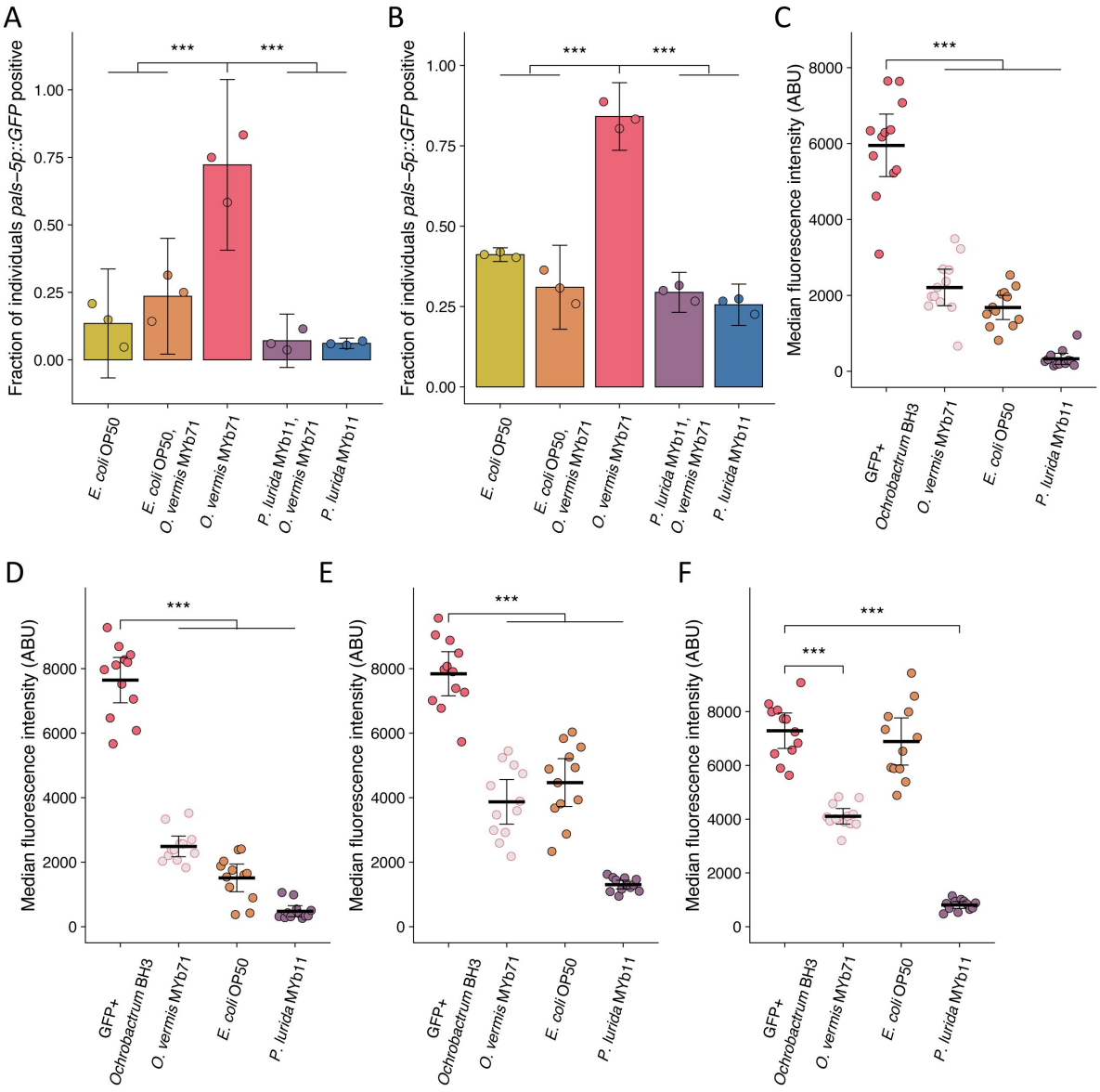
(A-B) The fraction of individuals *pals-5p::GFP* positive following exposure to 100 a.u. of exogenous Orsay virus. Data are from a single representative experiment and dots represent individual plates ((A) n = 1957 in total and n > 33 for all dots, (B) n = 1730 in total and n > 24 for all dots). The 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).



Supplementary Figure 11

Replicate susceptibility assays shown in Figure 6B and 6E.

(A-D) The fraction of individuals *pals-5p::GFP* positive following exposure to 10 a.u. exogenous Orsay virus in the presence of wild-type *P. lurida* MYb11 versus the indicated mutants. Data are from a single representative experiment and dots represent individual plates ((A) n = 681 in total and n > 39 for all dots, (B) n = 751 in total and n > 41 for all dots, (C) n = 2129 in total and n > 106 for all dots, (D) n = 1466 in total and n > 28 for all dots). For all plots the bar is the mean and error bars are the 95% confidence interval (C.I.). A-B) p-values determined using Student's t-test. (C-D) p-values determined using one-way ANOVA followed by Dunnett's test(NS non-significant, *p<0.05, **p<0.01, ***p<0.001).



Supplementary Figure 12

Replicate assays shown in Figure 7A, 7B, and 7C.

(A-B) The fraction of individuals *pals-5p::GFP* positive following exposure to 1 a.u. exogenous Orsay virus in the presence of pure lawns of the indicated bacteria or mixed lawns consisting of 50% of the indicated bacteria mixed with 50% *O. vermis* MYb71 (v:v, Methods). Data are from a single representative experiment. Bars represent mean and dots represent individual plates ((A) n=886 in total and n > 43 for all dots, (B) n=833 in total and n > 30 for all dots). **(C-F)** The median fluorescence observed in the intestinal lumen of individuals following exposure for (C-D) 4 h or (E-F) 24 h to pure GFP expressing *Ochrobactrum* BH3 lawns or mixed lawns consisting of 50% of the indicated bacteria mixed with 50% GFP expressing *Ochrobactrum* BH3 (v:v, Methods). Data are from a single representative experiment. Bars represent mean, dots represent individual animals. For all plots error bars represent 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,

Strain Name	Genotype	Source
WUM29	*virIs1[pHIP::Orsay-RNA1;pHIP::Orsay-RNA2;myo-2p::YFP];rde-1(ne219);jyIs8(pasl-5p::gfp;myo-2p::mCherry)*	Gift of Wang lab[1]
ERT54	*jyIs8[pals-5p::GFP;myo-2p::mCherry]*	CGC
WUM31	*rde-1(ne219);jyIs8[pals-5p::GFP;myo-2p::mCherry]*	Gift of Wang lab[2]
SS104	*glp-4(bn-2)*	CGC
ZD2610	*rde-1(ne219);jyIs8[pals-5p::GFP;myo-2p::mCherry]; glp-4(bn-2)*	This study
ZD2611	*jyIs8[pals-5p::GFP;myo-2p::mCherry]; glp-4(bn-2)*	This study
WUM104	*virEx[pHIP::RNA1WT-1]*	Gift of Wang lab[2]
WUM106	*virEx[pHIP::RNA1D601A-1]*	Gift of Wang lab[2]

Supplementary Table 1

List of *Caenorhabditis elegans* strains used in this study.

Supplementary Table 2

List of bacterial strains used in this study.

Strain Name	Genotype	Source
Escherichia coli OP50	WT	Kim Lab
Enterobacter xiangfangensis CEN2ent1	WT	CGC[3]
Lelliottia amnigena JUb66	WT	CGC[3]
Pseudomonas mendocina MSPm1	WT	CGC[3]
Comamonas piscis BIGb0172	WT	CGC[3]
Pantoea sp. BIGb0393	WT	CGC[3]
Providencia sp. JUb39	WT	Gift of Félix Lab[4]
Klebsiella sp. JUb268	WT	Gift of Félix Lab – unpublished
Achromobacter sp. JUb24	WT	Gift of Félix Lab[4]
Ochrobactrum vermis MYb71	WT	CGC[3]
Ochrobactrum BH3	WT	Gift of Troemel Lab[5]
GFP + Ochrobactrum BH3	WT(GFP+)	Gift of Troemel Lab[5]
Pseudomonas lurida MYb11	WT	CGC[3]
Pseudomonas lurida MYb11	ΔgacA	This study
Pseudomonas lurida MYb11	ΔkinB	This study
Pseudomonas lurida MYb11	ΔprpC	This study
Pseudomonas lurida MYb11	ΔptsP	This study
Pseudomonas aeruginosa PA01	WT	Gift of Dove Lab
Pseudomonas aeruginosa PA01	ΔlasI	Gift of Greenberg Lab[6]
Pseudomonas aeruginosa PA01	ΔlasR	Gift of Greenberg Lab[6]
Pseudomonas aeruginosa PA01	ΔrhlI	Gift of Greenberg Lab[6]
Pseudomonas aeruginosa PA01	ΔrhlR	Gift of Greenberg Lab[6]
Pseudomonas aeruginosa PA01	ΔpqsA	Gift of Dove Lab - unpublished
Pseudomonas aeruginosa PA01	ΔgacA	Gift of Dove Lab[7]
Pseudomonas aeruginosa PA01	ΔpelI	Gift of Parsek Lab[8]
Pseudomonas aeruginosa PA01	ΔpslI	Gift of Parsek Lab[9]
Pseudomonas aeruginosa PA01	ΔalgD	Gift of Parsek Lab[10]
Pseudomonas aeruginosa PA14	WT	Kim Lab
Pseudomonas aeruginosa PA14	ΔlasI	Gift of E. Drenkard and Ausubel Lab – Not published
Pseudomonas aeruginosa PA14	ΔlasR	Gift of E. Drenkard and Ausubel Lab[11]
Pseudomonas aeruginosa PA14	ΔrhlI	Gift of E. Drenkard and Ausubel Lab[12]
Pseudomonas aeruginosa PA14	ΔrhlR	Gift of E. Drenkard and Ausubel Lab[11]
Pseudomonas aeruginosa PA14	ΔpqsE	Gift of Paczkowski Lab[13]
Pseudomonas aeruginosa PA14	ΔrhlI;ΔpqsE	Gift of Paczkowski Lab[13]
Pseudomonas aeruginosa PA14	ΔgacA	Gift of Pukkila-Worley Lab[14]
Pseudomonas aeruginosa PA14	ΔpelA	Gift of E. Drenkard and Ausubel Lab[15]
Pseudomonas aeruginosa PA14	ΔalgD	Gift of E. Drenkard and Ausubel Lab[16]
Pseudomonas aeruginosa PA14	Mutants from the Nonredundant PA14 Transposon Insertion Library	E. Drenkard and Ausubel Lab[14]

Bold and underlined indicate mutants with confirmed phenotype.

Identifier	Identifier	Regulon	Mutant ID
PA14_39800	fecI	gacA	5297
PA14_63160	pmrB	gacA	5751
PA14_19020	tse3	gacA	6047
PA14_43900		Virulence	6472
PA14_55170	cat	gacA	6692
PA14_42340	pscD	gacA	6764
PA14_06750	nitrite reductase;protein_id=QDL62541.1	RhlR	6811
PA14_09660		gacA	23248
PA14_43050	PA01-tssA1	gacA	23266
PA14_42470	pcrV	gacA	23320
PA14_19100	alpha/beta hydrolase;protein_id=QDL63509.1	RhlR	23291
PA14_12030		Virulence	23465
PA14_23430		Virulence	23782
PA14_09300		Virulence	23790
PA14_09300		gacA	23790
PA14_52690		Virulence	24118
PA14_46680	norM	gacA	24085
PA14_06720	protein nirF;protein_id=QDL62538.1	RhlR	24165
PA14_15080		gacA	24311
PA14_26230	hisQ	gacA	24643
PA14_28130	bswR	gacA	24728
PA14_21000	hypothetical protein;protein_id=QDL63663.1	RhlR	25384
PA14_21000	hypothetical protein;protein_id=QDL63663.1	RhlR	25384
PA14_15200		gacA	25712
PA14_42950		Virulence	25708
PA14_36290	NADP-dependent oxidoreductase;protein_id=QDL64899.1	RhlR	25752
PA14_06180	fecI	gacA	25794
PA14_58030	fumC1	gacA	25811
PA14_40310	acyl carrier protein;protein_id=QDL65222.1	RhlR	26047
PA14_39440		gacA	26130
PA14_39790		gacA	26678
PA14_42620	pscR	gacA	26713
PA14_70720		gacA	26837
PA14_52230	pirA	gacA	27108
PA14_14850		Virulence	27116
PA14_37260	OprD family porin;protein_id=QDL64986.1	RhlR	27286
PA14_55580	hemO	gacA	27752
PA14_53910	No clear ortholog	gacA	27823
PA14_48520	transglycosylase;protein_id=QDL65879.1	RhlR	27859
PA14_36310	cyanide-forming glycine dehydrogenase subunit HcnC;protein_id=QDL64901.1	RhlR	28000
PA14_21680		gacA	28071
PA14_07330		gacA	28734
PA14_10200	fvbA	gacA	28811
PA14_31370	methyltransferase domain-containing protein;protein_id=QDL64513.1	RhlR	28925
PA14_01150	eagt6	gacA	28943
PA14_09690	bfiS	gacA	28988
PA14_38950		gacA	29098
PA14_21020	non-ribosomal peptide synthetase;protein_id=QDL63665.1	RhlR	29306
PA14_09630		gacA	29508

Supplementary Table 3

List of *P. aeruginosa* PA14 transposon insertion mutants screened in this study.

PA14_56090		gacA	29512
PA14_42350	pscC	gacA	29579
PA14_20960	nuclear transport factor 2 family protein;protein_id=QDL63660.1	RhlR	29854
PA14_04340		gacA	29941
PA14_38850	exaB	gacA	30227
PA14_60650		gacA	30214
PA14_01140	tse6	gacA	30560
PA14_64920	methyl-accepting chemotaxis protein;protein_id=QDL67201.1	RhlR	30889
PA14_65170	rpsR	gacA	31176
PA14_58570		gacA	31335
PA14_52670		Virulence	31424
PA14_29650	psl and pyoverdine operon regulator PpyR;protein_id=QDL64369.1	RhlR	31563
PA14_43350		Virulence	31640
PA14_02910	IclR family transcriptional regulator;protein_id=QDL62237.1	RhlR	31642
PA14_42400	exsB	gacA	31713
PA14_47380	hxuA	gacA	31886
PA14_00890	pppA	gacA	31907
PA14_52660		Virulence	32331
PA14_58040		gacA	32484
PA14_23000	carbohydrate ABC transporter permease;protein_id=QDL63835.1	RhlR	32514
PA14_58250	magD	gacA	32575
PA14_27700		Virulence	32578
PA14_14340		gacA	33029
PA14_28895		gacA	33357
PA14_63770	Also hits PA14_63760	gacA	33569
PA14_31730		gacA	33612
PA14_31350	NAD(P)/FAD-dependent oxidoreductase;protein_id=QDL67941.1	RhlR	33671
PA14_69810	**glnK**	**Virulence**	**33817**
PA14_34130	icmF3	gacA	34003
PA14_25690	**fabF1**	**Virulence**	**34095**
PA14_33260	pvdS	gacA	34241
PA14_22990		gacA	34541
PA14_42450	popB	gacA	34677
PA14_42440	popD	gacA	34677
PA14_30650	**gacA**	**Virulence**	**34781**
PA14_31580		Virulence	34827
PA14_34050	hsiC3	gacA	34884
PA14_58010		gacA	34966
PA14_71100	YeiH family protein;protein_id=QDL67678.1	RhlR	35049
PA14_71840		gacA	35192
PA14_36300	TetR/AcrR family transcriptional regulator;protein_id=QDL64900.1	RhlR	35194
PA14_40290	protease LasA;protein_id=QDL65220.1	RhlR	35267
PA14_28350	alpha/beta hydrolase;protein_id=QDL64257.1	RhlR	35370
PA14_09350		gacA	35518
PA14_72390	**kinB**	**Virulence**	**35639**
PA14_09320		Virulence	35711
PA14_38770	pqqH	gacA	35762
PA14_23460		Virulence	35855
PA14_52130	wzz2	gacA	35858
PA14_05960		Virulence	36116
PA14_20580	aliphatic amidase expression-regulating protein;protein_id=QDL63629.1	RhlR	36129
PA14_04410	**ptsP**	**Virulence**	**36275**
PA14_58000	sodM	gacA	36454
PA14_63220		gacA	36489

Supplementary Table 3 (continued)

PA14_37250	MFS transporter;protein_id=QDL64985.1	RhlR	36598
PA14_38820	pqqB	gacA	36607
PA14_01030	hcp1	gacA	36995
PA14_01030	hcp3	gacA	36995
PA14_00560	exoT	gacA	36955
PA14_07360		gacA	36956
PA14_00080		gacA	37111
PA14_11660	aqpZ	gacA	37239
PA14_00580		gacA	37604
PA14_54640		Virulence	37629
PA14_16160		gacA	37763
PA14_69000		Virulence	37818
PA14_19120	**RhlR**	**Virulence**	**37943**
PA14_38900	eraR	gacA	38131
PA14_14330	spcS	gacA	38258
PA14_49750	MFS transporter;protein_id=QDL65983.1	RhlR	38758
PA14_20970	cytochrome P450;protein_id=QDL63661.1	RhlR	38837
PA14_38440		Virulence	38983
PA14_53940		Virulence	39064
PA14_45940		Virulence	39292
PA14_30230	**clpA**	**Virulence**	**39351**
PA14_11120	response regulator transcription factor;protein_id=QDL62884.1	RhlR	39369
PA14_62280	hmuV	gacA	39440
PA14_00820	tagQ1	gacA	39485
PA14_39000		gacA	39587
PA14_02140	siaB	gacA	39659
PA14_38190		gacA	39754
PA14_20190	nitrous oxide reductase family maturation protein NosD;protein_id=QDL63598.1	RhlR	39889
PA14_34110	dotU3	gacA	40266
PA14_52580		Virulence	40436
PA14_35130	ArsR family transcriptional regulator;protein_id=QDL64805.1	RhlR	40419
PA14_53950	**prpC**	**Virulence**	**41035**
PA14_38840	exaC	gacA	41052
PA14_32880		gacA	41110
PA14_42430	exsC	gacA	41125
PA14_28000		gacA	41577
PA14_38200		gacA	41886
PA14_22980	carbohydrate ABC transporter substrate-binding protein;protein_id=QDL63833.1	RhlR	42223
PA14_36330	hcnA	gacA	42284
PA14_01010	hsiB1	gacA	42268
PA14_01010	hsiB2	gacA	42268
PA14_01010	hsiB3	gacA	42268
PA14_63120	spermidine synthase;protein_id=QDL68019.1	RhlR	42429
PA14_63120	speE2	gacA	42429
PA14_38780	pqqE	gacA	42390
PA14_00940	tssK1	gacA	42462
PA14_67530		gacA	42570
PA14_68730		Virulence	42600
PA14_05310		Virulence	42799
PA14_16180		gacA	42990
PA14_56890	mexW	gacA	43547
PA14_63800	mgtA	gacA	43710
PA14_31720		gacA	43724
PA14_15990	trmD	gacA	43735

Supplementary Table 3 (continued)

PA14_29660	short-chain dehydrogenase;protein_id=QDL64370.1	RhlR	43837
PA14_21030	ATP-dependent Clp protease proteolytic subunit;protein_id=QDL63666.1	RhlR	44009
PA14_63130		gacA	44414
PA14_20200	nitrous-oxide reductase;protein_id=QDL63599.1	RhlR	44533
PA14_18860	No clear ortholog	gacA	44571
PA14_42270	pscJ	gacA	45034
PA14_38220		gacA	45228
PA14_09970	fpvB	gacA	45188
PA14_51380		Virulence	45262
PA14_47110		gacA	45355
PA14_38860	exaA	gacA	45932
PA14_61190	cdrB	gacA	45954
PA14_51250		gacA	46490
PA14_24990		gacA	46692
PA14_62810	secG	gacA	47247
PA14_48590		Virulence	47923
PA14_49740	hypothetical protein;protein_id=QDL67981.1	RhlR	48441
PA14_49740		gacA	48441
PA14_01020	hsiC2	gacA	48448
PA14_63580	fdnH	gacA	48501
PA14_42460	pcrH	gacA	48586
PA14_62290	phuU	gacA	48599
PA14_08360	trpC	gacA	48714
PA14_20230	regulatory protein NosR;protein_id=QDL63600.1	RhlR	48720
PA14_08370		Virulence	52692
PA14_42380	exsD	gacA	52698
PA14_30580		Virulence	52787
PA14_57990		gacA	52805
PA14_22020		Virulence	52889
PA14_62350	phuR	gacA	53271
PA14_41710		Virulence	53876
PA14_36350		gacA	54275
PA14_40030		gacA	54445
PA14_52840		gacA	54510
PA14_64940	nicotinate-nicotinamide nucleotide adenylyltransferase;protein_id=QDL67203.1	RhlR	54555
PA14_16270		gacA	54625
PA14_53640		gacA	54847
PA14_20020	hasAp	gacA	55211
PA14_33050		gacA	55625
PA14_39990		gacA	55689
PA14_02110	siaD	gacA	55715
PA14_41730		Virulence	55833
PA14_20920	class I SAM-dependent methyltransferase;protein_id=QDL63657.1	RhlR	55954
PA14_38800	pqqC	gacA	56080
PA14_01120	tsi6	gacA	56172
PA14_01100	clpV1	gacA	56461
PA14_52720		Virulence	56790
PA14_72050		gacA	57004
PA14_52260		Virulence	42741
PA14_61680		Virulence	46240
PA14_38970	ercS'	gacA	1242
PA14_31700		gacA	738
PA14_42280	pscI	gacA	383

Supplementary Table 3 (continued)

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Editors

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Senior Editor
Wendy Garrett
Harvard T.H. Chan School of Public Health, Boston, United States of America

Reviewer #1 (Public Review):

Summary:

This manuscript explores the importance of food type on virus infection dynamics using a nematode virus as a model system. The authors demonstrate that susceptibility to viral infection can change by several orders of magnitude based on the type of bacterial food that potential hosts consume. They go on to show that, for the bacterial food source that reduces susceptibility, the effect is modulated by quorum sensing molecules that the bacteria produce.

Strengths:

This manuscript shows convincingly that nematode susceptibility to viral infection changes by several orders of magnitude (i.e. doses must be increased by several orders of magnitude to infect the same fraction of the population) depending on the bacterial food source on which hosts are reared. The authors then focus on the bacteria that reduce host susceptibility to viral infection and demonstrate that certain bacterial quorum-sensing compounds are required to see this effect of reduced susceptibility. Overall, sample sizes are large, methods are generally rigorous, experiments are repeated, and patterns are clear.

Comments on revised version:

The authors have now addressed all of my previous concerns.

https://doi.org/10.7554/eLife.92534.2.sa1

Reviewer #2 (Public Review):

In this study, the authors investigate how diverse bacterial species influence Orsay virus transmission and host susceptibility in *C. elegans*. They find that Ochrobactrum species increase infection rates, while Pseudomonas species decrease infection rates, and they identify regulators of quorum sensing and the gacA two-component system as genetic factors in the effects of Pseudomonas on infection. These findings provide important insights into the species-specific effects that bacteria can have on viral infection in *C. elegans*, and they may have relevance for the impact of bacterial species on viral infection in other systems. Overall the manuscript has high rigor. However, a few minor concerns are listed below.

(1) The authors state that the amount of bacteria added to each plate was standardized by seeding plates with equivalent volumes of overnight culture. This approach does not account for differences in bacterial growth rate. A more rigorous approach would be to standardize based on OD600 measurements or CFU's. Alternatively, the authors could include bacterial growth curves to demonstrate that each strain/species has reached a similar growth phase (i.e. late log) at the time of plating, as bacterial physiology and virulence is dependent on the stage of growth. At the least, if it is not possible to perform these experiments, it would be useful to include a statement that potential differences in bacterial growth rate may influence their conclusions.

(2) Line 314-315: The claim "We did not observe any potent effect on host susceptibility to infection by Orsay virus from any supernatant (Supp. Fig. 9)" is not fully supported by the data, as the data in Fig S9 only show pals-5p::GFP levels. To confirm that host susceptibility is not affected, the authors would also measure the viral infection rate and/or viral load. Otherwise, the authors should rephrase the conclusion to increase accuracy. For example, "We did not observe any potent effect on pals-5p::GFP activation upon Orsay virus infection when animals were exposed to bacterial culture supernatant".

(3) The Ct values shown in Fig 3B-F should be normalized to a reference gene (i.e. Ct values for snb-1).

https://doi.org/10.7554/eLife.92534.2.sa0

Author response:

The following is the authors' response to the original reviews.

> ***eLife assessment***
>
> *This important study identifies differential Orsay virus infection of C. elegans when animals are fed on different bacteria. The evidence for this is however, incomplete, as experiments to control for feeding rate and bacterial pathogenicity are needed as well as direct quantification of viral load.*

We appreciate that the editors and reviewers felt that our manuscript addressed an important problem. We appreciate the constructive critiques provided by the reviewers and have worked to address all of the concerns, including a number of additional experiments as indicated below.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *This manuscript explores the importance of food type on virus infection dynamics using a nematode virus as a model system. The authors demonstrate that susceptibility to viral infection can change by several orders of magnitude based on the type of bacterial food that potential hosts consume. They go on to show that, for the bacterial food source that reduces susceptibility, the effect is modulated by quorum sensing molecules that the bacteria produce.*
>
> *Strengths:*
>
> *This manuscript shows convincingly that nematode susceptibility to viral infection changes by several orders of magnitude (i.e. doses must be increased by several orders of magnitude to infect the same fraction of the population) depending on the bacterial food source on which hosts are reared. The authors then focus on the bacteria that reduce host susceptibility to viral infection and demonstrate that certain bacterial quorum-sensing compounds are required to see this effect of reduced susceptibility. Overall, sample sizes are large, methods are generally rigorous, experiments are repeated, and patterns are clear.*
>
> *Weaknesses:*
>
> *Although the molecular correlate of reduced susceptibility is identified (i.e. quorum sensing compounds) the mechanisms underlying this effect are missing. For example, there are changes in susceptibility due to altered nutrition, host condition, the microbiome, feeding rate, mortality of infected hosts, etc. In addition, the authors focus almost entirely on the reduction in susceptibility even though I personally find the increased susceptibility generated when reared on Ochrobactrum to be much more exciting.*

> *I was a bit surprised that there was no data on basic factors that could have led to reductions in susceptibility. In particular, data on feeding rates and mortality rates seem really important. I would expect that feeding rates are reduced in the presence of Pseudomonas. Reduced feeding rates would translate to lower consumed doses, and so even though the same concentration of virus is on a plate, it doesn't mean that the same quantity of virus is consumed. Likewise, if Pseudomonas is causing mortality of virus-infected hosts, it could give the impression of lower infection rates. Perhaps mortality rates are too small in the experimental setup to explain this pattern, but that isn't clear in the current version of the manuscript. Is mortality greatly impacted by knocking out quorum-sensing genes? Also, the authors explored susceptibility to infection, but completely ignored variation in virus shedding.*

We have added data on feeding rates (Line numbers 141-148 and 176-182, Supplementary Figure 4). After six hours of exposure no differences in feeding rate were observed. After 24 hours minor differences emerged between O. vermis MYb71 and each Pseudomonas species, however feeding rate inversely correlated with susceptibility to Orsay virus in that O. vermis MYb71 displayed the lowest feeding rate while P. aeruginosa PA14 displayed the highest feeding rate.

We have also added data on mortality rates (Line numbers 183-200, Supplementary Figure 6). No significant mortality was observed within the 24-hour exposure period used for our Orsay infection and transmission assays. P. aeruginosa virulence is dependent upon temperature and as our assays are done at 20°C rather than 25°C this may account for reduced mortality compared to other published results. Regardless, we noted that O. vermis MYb71 killed C. elegans as quickly as P. aeruginosa PA14 under these conditions and these two bacteria led to the shortest lifespan compared to the other tested bacteria. Interestingly, P. lurida MYb11 was observed to be more virulent than P. aeruginosa PA01 under these conditions. These results suggest that there is no direct correlation between mortality and susceptibility to Orsay virus, although it does not rule out that virulence effects unique to each bacterium could contribute to alterations in host susceptibility.

The reviewer is correct to assert that differences in viral shedding could exist. However, our susceptibility assays using exogenous Orsay virus remove this source of variation and yet we still observe the same trends such that O. vermis MYb71 promotes infection while P. lurida MYb11, P. aeruginosa PA01, and P. aeruginosa PA14 attenuate infection. Further we measured the amount of virus shed into the lawns in the presence of different bacteria and did not observe differences in shed virus that could account for the differences we observe in incidence proportion (Line numbers 241-254, Fig. 3 F). Viral stability could be an issue in both the transmission and susceptibility assays. We therefore tested viral stability in the presence of E. coli, P. lurida MYb11, P. aeruginosa PA01, and P. aeruginosa PA14 and successfully recovered virus from all lawns, suggesting virus is not rapidly degraded in the presence of any bacterium (Fig. 3D and 3E). However, we noted that the recovery of Orsay virus from lawns of E. coli OP50 and P. lurida MYb11 within 30 minutes was decreased compared to a spike-in control suggesting recovery from each lawn is not equivalent. This complicates a comparison of viral stability and shedding rates between different bacteria, but our ability to recover substantial amounts of virus in the shedding assay from the three Pseudomonas strains we examined precludes a substantial decrease in shedding rates as an explanation for the robust attenuation of Orsay virus observed in transmission assays.

> *I was also curious why the authors did not further explore the mechanism behind the quorumsensing effect. Not sure whether this is possible, but would it be possible to add spent media to the infection plates where the spent media was from Pseudomonas that produce the quorum sensing compound but the plates contain OP50, Pseudomonas, or*

> *the quorum sensing knockout of Pseudomonas? That would reveal whether it is the compound itself vs. something that the compound does.*

We observed that quorum sensing mutants suppressed the attenuation of Orsay virus infection and we agree that this could be a consequence of the compounds themselves, or more likely an effect of the downstream consequences of quorum signaling. We added culture supernatant from each bacterium to lawns of E. coli OP50 to assess the effect on host susceptibility and did not observe any potent effect (Line numbers 311-318, Supplementary Figure 9). This supports an interpretation that it is not the compound itself that is responsible, however we cannot rule out that the compounds themselves may be responsible if provided at a higher concentration.

> *In addition, I was surprised by how much focus there was on the attenuation of infection and how little there was on the enhancement of infection. To me, enhancement seems like the more obvious thing to find a mechanism for -- is the bacteria suppressing immunity, preventing entry to gut cells, etc?*

We are also intrigued by the enhancement of infection by Ochrobactrum spp, however we chose to focus on attenuation given the availability of Pseudomonas aeruginosa genetic mutants for study. We have added data (Line numbers 371-402, Figure 7, and Supplemental Figure 12) that inform our current hypothesis regarding Ochrobactrum mediated enhancement of Orsay virus infection.

> *I was a bit concerned about the "arbitrary units", which were used without any effort to normalize them. David Wang and Hongbing Jiang have developed a method based on tissue culture infectious dose 50 (TCID50) that can be used to measure infectious doses in a somewhat repeatable way. Without some type of normalization, it is hard to imagine how this study could be repeated. The 24-hour time period between exposure and glowing suggests very high doses, but it is still unclear precisely how high. Also, it is clear that multiple batches of virus were used in this study, but it is entirely unclear how variable these batches were.*

We have clarified that we also measured the (TC)ID50 for every batch of virus used similar to the methods suggested by the Wang laboratory (Line numbers 107-119 and 499-506). We have added a figure showing the virus batch variability for all batches used in this study (Supp. Fig. 2). We have further clarified that the arbitrary units correspond to the actual microliters of viral filtrate used during infection and provided clear methods to replicate our viral batch production to assist with issues of reproducibility (Line numbers 107-119 and 499-506).

> *The authors in several places discuss high variability or low variability in incidence as though it is a feature of the virus or a feature of the host. It isn't. For infection data (or any type of binomial data) results are highly variable in the middle (close to 50% infection) and lowly variable at the ends (close to 0% or 100% infection). This is a result that is derived from a binomial distribution and it should not be taken as evidence that the bacteria or the host affect randomness. If you were to conduct dose-response experiments, on any of your bacterial food source treatments, you would find that variability is lowest at the extremely high and extremely low doses and it is most variable in the middle when you are at doses where about 50% of hosts are infected.*

Thank you for pointing this out, we have removed all reference to this throughout the manuscript.

Reviewer #2 (Public Review):

Summary and Major Findings/Strengths:

Across diverse hosts, microbiota can influence viral infection and transmission. C. elegans is naturally infected by the Orsay virus, which infects intestinal cells and is transmitted via the fecal-oral route. Previous work has demonstrated that host immune defense pathways, such as antiviral RNAi and the intracellular pathogen response (IPR), can influence host susceptibility to virus infection. However, little is known about how bacteria modulate viral transmission and host susceptibility.

In this study, the authors investigate how diverse bacterial species influence Orsay virus transmission and host susceptibility in C. elegans. When C. elegans is grown in the presence of two Ochrobactrum species, the authors find that animals exhibit increased viral transmission, as measured by the increased proportion of newly infected worms (relative to growth on E. coli OP50). The presence of the two Ochrobactrum species also resulted in increased host susceptibility to the virus, which is reflected by the increased fraction of infected animals following exposure to the exogenous Orsay virus. In contrast, the presence of Pseudomonas lurida MYb11, as well as Pseudomonas PA01 or PA14, attenuates viral transmission and host susceptibility relative to E. coli OP50. For growth in the presence of P. aeruginosa PA01 and PA14, the attenuated transmission and susceptibility are suppressed by mutations in regulators of quorum sensing and the gacA two-component system. The authors also identify six virulence genes in P. aeruginosa PA14 that modulate host susceptibility to virus and viral transmission, albeit to a lesser extent. Based on the findings in P. aeruginosa, the authors further demonstrate that deletion of the gacA ortholog in P. lurida results in loss of the attenuation of viral transmission and host susceptibility.

Taken together, these findings provide important insights into the species-specific effects that bacteria can have on viral infection in C. elegans. The authors also describe a role for Pseudomonas quorum sensing and virulence genes in influencing viral transmission and host susceptibility.

Major weaknesses:

The manuscript has several issues that need to be addressed, such as insufficient rigor of the experiments performed and questions about the reproducibility of the data presented in some places. In addition, confounding variables complicate the interpretations that can be made from the authors' findings and weaken some of the conclusions that are stated in the manuscript.

(1) The authors sometimes use pals-5p::GFP expression to indicate infection, however, this is not necessarily an accurate measure of the infection rate. Specifically, in Figures 4-6, the authors should include measurements of viral RNA, either by FISH staining or qRT-PCR, to support the claims related to differences in infection rate.

Following the reviewers comment we have corroborated our pals-5::GFP data using FISH staining (Line numbers 291-292 and 357-359, Figure 4D & 4E, and Figure 6C).

(2) In several instances, the experimental setup and presentation of data lack sufficient rigor. For example, Fig 1D and Fig 2B only display data from one experimental replicate. The authors should include information from all 3 experimental replicates for more transparency. In Fig 3B, the authors should include a control that demonstrates how RNA1 levels change in the presence of E. coli OP50 for comparison with the results showing replication in the presence of PA14. In order to support the claim that "P. aeruginosa and P. lurida MYb11 do not eliminate Orsay virus infection", the authors

> *should also measure RNA1 fold change in the presence of PA01 and P. lurida in the context of exogenous Orsay virus. Additionally, the authors should standardize the amount of bacteria added to the plate and specify how this was done in the Methods, as differing concentrations of bacteria could be the reason for species-specific effects on infection.*

All experimental replicates are now included within the supplementary information.

We have also measured RNA1 fold change following infection in the presence of P. aeruginosa PA01 and P. lurida MYb11 (Line numbers Fig 3B and 3C) and found that these bacteria also do not eliminate Orsay virus replication.

We thank the reviewer for their comment on controlling the amount of bacteria and have clarified our methods section to more clearly explain that we seed our plates with equivalent amounts (based on volume) of overnight bacterial culture before allowing the bacteria to grow on the plates for 48 hours.

> *(3) The authors should be more careful about conclusions that are made from experiments involving PA14, which is a P. aeruginosa strain (isolated from humans), that can rapidly kill C. elegans. To eliminate confounding factors that are introduced by the pathogenicity of PA14, the authors should address how PA14 affects the health of the worms in their assays. For example, the authors should perform bead-feeding assays to demonstrate that feeding rates are unaffected when worms are grown in the presence of PA14. Because Orsay virus infection occurs through feeding, a decrease in C. elegans feeding rates can influence the outcome of viral infection. The authors should also address whether or not the presence of PA14 affects the stability of viral particles because that could be another trivial reason for the attenuation of viral infection that occurs in the presence of PA14.*

We have added data on feeding rates (Line numbers 141-148 and 176-182, Supplementary Figure 4). After six hours of exposure no differences in feeding rate were observed. After 24 hours minor differences emerged between O. vermis MYb71 and each Pseudomonas species, however feeding rate inversely correlated with susceptibility to Orsay virus in that O. vermis MYb71 displayed the lowest feeding rate while P. aeruginosa PA14 displayed the highest feeding rate.

We have also added data on mortality rates (Line numbers 183-200, Supplementary Figure 6). No significant mortality was observed within the 24-hour exposure period used for our Orsay infection and transmission assays. P. aeruginosa virulence is dependent upon temperature and as our assays are done at 20°C rather than 25°C this may account for reduced mortality compared to other published results. Regardless, we noted that O. vermis MYb71 killed C. elegans as quickly as P. aeruginosa PA14 under these conditions and these two bacteria led to the shortest lifespan compared to the other tested bacteria. Interestingly, P. lurida MYb11 was observed to be more virulent than P. aeruginosa PA01 under these conditions. These results suggest that there is no direct correlation between mortality and susceptibility to Orsay virus, although it does not rule out that virulence effects unique to each bacterium could contribute to alterations in host susceptibility.

We tested viral stability in the presence of E. coli OP50 and Pseudomonas spp. and successfully recovered virus from all lawns, suggesting virus is not rapidly degraded in the presence of P. lurida MYb11, P. aeruginosa PA01, and P. aeruginosa PA14 (Line numbers 241-249, Fig 3D and Fig 3E). However, we noted that the recovery of Orsay virus from lawns of E. coli OP50 and P. lurida MYb11 within 30 minutes was decreased compared to a spike-in control suggesting recovery from each lawn is not equivalent. This complicates a comparison of viral stability and shedding rates between different bacteria, but our ability to recover

substantial amounts of virus in the shedding assay from each Pseudomonas species precludes a substantial decrease in shedding rates as an explanation for the robust attenuation of Orsay virus observed in transmission assays.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *Overall, I really liked this manuscript, I do think there are areas for improvement though.*
>
> *Some smaller things:*
>
> *Line 84: "can be observed spreading from a single animal" -- this isn't really great wording because the virus itself can't be observed (at least not very easily) -- even infection is hard to see.*

The wording in line 84-85 has now been adjusted to read “can spread from a single animal”.

> *Fig 1C: which groups are statistically significantly different from each other?*

Statistics have now been added to Figure 1C.

> *Line 154: not necessary to do for this paper, but this sentence made me curious whether the effect would have been seen with mixtures of bacteria (i.e. what if 50% were OP50 and 50% were Pseudomonas?)*

This data has now been added in Line numbers 372-378, Figure 7A, and Supp. Fig. 12A and 12B.

> *Line 262-264: I don't find this interesting at all for the reasons mentioned earlier about binomial data being the most variable in the middle.*

These lines have been removed.

> *Figure 4 B: The labels for the first two tick marks on the x-axis are switched I suspect. Otherwise, the controls did not behave as expected.*

Figure 4B has been corrected.

> *Line 288, 297 and several other places: "Orsay Virus" should be "Orsay virus".*

We have corrected these instances.

> *Supplemental Figure 2: Labels in the figure legend are B and C instead of A and B.*

These labels have been adjusted for their placement within Figure 6.

> *Line 411: I suspect this was supposed to be 13,200 xg rather than 13.2 xg.*

This error has been corrected.

> *Line 416-417: This sentence is very hard to interpret. More details are needed. This is the ID50 in which host strain? Is this averaged over all batches of virus? How variable are the batches?*

This sentence (line number 114) has been amended to clarify that all ID50 values referred to here were calculated for ZD2611 populations in the presence of E. coli OP50. Further, Supplementary Figure 2 now shows all the ID50 values measured for each batch of virus used in this manuscript resulting in an average ID50 of 3.6.

> *Lines 467-469: Why exclude these instead of counting them as zeros in the analysis? How many plates fit this description -- were there lots or only a few over the course of all experiments?*

We have chosen to exclude these plates as these samples lost spreaders at some point during the course of the assay potentially skewing the eventual number of new infections counted depending on when the infected spreader animal crawled off the plate. We have detailed the number of plates that fit this description in lines 559-562.

> *Line 476: A critical detail that is missing here is what number of worms were counted to score infection. Please say here or in the figure legends.*

We have added the total number of worms counted and the minimum number counted per plate for each assay in the figure legends.

> *Line 546: Why was only a single representative experiment shown? I'm asking for a justification, not necessarily for you to show all the data.*

We chose to show a single representative experiment for two reasons: We noted variability between susceptibility assays even when using the same batch of virus such that we could not combine experiments into a single plot as we did for transmission assays. Second, while we could normalize to a control within each experiment and expect to see similar relative differences across experiments, we believe this makes it more difficult to interpret the underlying data. For example, an increase in the infection rate of 80% compared to 10% within a population has only a single interpretation while a relative increase in the infection rate by 8x within a population could have several underlying meanings (e.g. 80% vs 10%, 64%vs 8%, 24% vs 3%). We have now included all experimental replicates in the supplementary material.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *Minor concerns:*
>
> *(1) Lines 86-87: "utilized a collection of bacteria isolated from the environment with wild C. elegans". The authors should provide more context on the source of these bacterial strains.*

More references for the sources of these bacteria have been added to Supplementary Table 2.

> *(2) The presentation of data in Fig 1 could be improved. The authors should include the text "pals-5p::GFP" on the images shown in Fig 1B. The red dashed line in Fig. 1D should intersect the dose-response curve at y = 0.5. The column heading for Fig 1E states "ID50 +/- SD (a.u.)", but should read "ID50 ratio" and should not have units. It also might be more intuitive to normalize the ID50 value for O. vermis to E. coli OP50. This way, having an ID50 ratio >1 indicates decreased transmission relative to E. coli, and ID50 ratio <1 indicates increased transmission relative to E. coli. To increase the transparency and rigor of 1E, the authors should plot the ratios from all 3 experimental replicates. The authors should also briefly explain why different viral doses were used in Fig 1D and 1F.*

The text “pals-5p::GFP” has now been added to Figure 1B and throughout the text. The red dashed line in figure 1D has been corrected. Figure 1E has been adjusted to an actual figure as suggested and the y-axis label is “ID50 Ratio Compared to E. coli OP50”. The ID50 replicates have been plotted in Supplementary Figure 2. We have clarified that the doses used are the same. Briefly, the technical replicates of individual doses from Figure 1D and Supplementary Figure 3A and 3B were pooled and processed for FISH staining to provide each experimental replicate of Figure 1F.

> *(3) Line 110: The claim is that Ochrobactrum and P. lurida MYb11 reduce the variability of infection levels. However, another possibility is that there's simply less dynamic range in the assay because the infection levels have been compressed to 100% and 0% under these conditions.*

This line has been removed.

> *(4) There are discrepancies between what is shown in Fig 2C and what is described in the text. Lines 163-164: "P. aeruginosa PA01 and P. lurida MYb11 attenuated average infection to 33% and 62% of the population respectively". In Fig 2C, the mean for PA01 is ~25% whereas the mean for P. lurida appears to be less than 62%.*

These values have been corrected.

> *(5) Line 196: Provide more context for why rde-1 mutants were tested. This is the first time rde-1 is mentioned in the text (i.e. why show results in rde-1 mutants when the results are in Fig 2).*

More context has been provided for why rde-1 mutants were tested (Line numbers 228-232). Briefly, using the rde-1 mutant, which has defective antiviral immunity and therefore supports higher viral replication levels than the wild-type (Félix et al. 2011), allows us to potentiate our infection assay in Figure 3B and 3C such that we maximize our chances of detecting viral replication in the presence of the Pseudomonas species, and especially P. aeruginiosa PA14, where fewer animals might be expected to get infected based upon Figure 2B and Supplementary Figure 5.

> *(6) Lines 228-229: "Mutations of any the regulators of the las, rhl, or pqs quorum sensing systems suppressed the attenuation of Orsay virus infection caused by the presence of wild-type P. aeruginosa PA01". Based on this description, PA01 should have a lower fraction of GFP positive relative to the quorum sensing mutants in Fig 4B. It seems that the x-axis labels OP50 and PA01 are swapped.*

The x-axis labels of Figure 4B have been corrected.

> *(7) To improve clarity, for any figures that have data showing the "fraction of individuals GFP positive", the authors should include "pals-5p::GFP" in the y-axis title and legend.*

The y-axis labels, legends, and text have been corrected throughout.

> *(8) To improve overall clarity and flow, the order in which the data is presented could be reordered. In particular, Fig. 6 could be better positioned instead of being the last figure, as no further characterization is performed on the mutants, and the findings are not conserved in strains that are more relevant to the C. elegans microbiota, such as P. lurida. The overall story could be strengthened if the authors ended the manuscript with*

> *more details related to the mechanism by which regulators of quorum sensing modulate the outcome of viral infection.*

Figure 5 and Figure 6 have now been swapped.

> *(9) Fig 5A: Make arrow sizes consistent across diagrams (i.e. the diagram for gacA deletion).*

This figure (now Figure 6A) has been adjusted to make arrow sizes consistent across diagrams.

> *(10) Lines 280-282: "These data suggest that gacA has a conserved role across distant Pseudomonas species..." Here, the authors can provide more context on how well-conserved gacA is across Pseudomonas species (i.e. phylogenetic analysis of gacA sequences across different Pseudomonas species/strains). Furthermore, the data in Fig 5 does not provide strong enough support for the conclusion that gacA has a conserved role broadly across Pseudomonas species, as the authors only assess the effects of a gacA deletion in two species, P. aeruginosa and P. lurida.*

We have adjusted lines 361-362 to “These data suggest that gacA has a conserved role between P. aeruginosa and P. lurida Myb11 in the attenuation of Orsay virus transmission and infection of C. elegans.” to reflect that we only assessed the effects of the gacA deletion in P. aeruginosa and P. lurida MYb11.

> *(11) The manuscript can be strengthened by performing additional experiments to elucidate the mechanism by which Pseudomonas modulates viral infection. Does the attenuation of viral transmission and host susceptibility by P. lurida and P. aeruginosa require C. elegans to be in the presence of live bacteria? For example, the authors could measure viral transmission and susceptibility of C. elegans grown on heat-killed Pseudomonas. Additionally, it would be interesting to determine if modulation of viral infection is dependent on a secreted molecule. To assess this, the authors could perform viral infections in the context of Pseudomonas culture supernatant.*

We added bacterial culture supernatant from each bacterium to lawns of E. coli OP50 to assess the effect on host susceptibility and did not observe any potent effect (Line numbers 311-318, Supplementary Figure 9). This supports an interpretation that attenuation is not mediated by a secreted molecule, however we cannot rule out that attenuation activity would become apparent if supernatant were provided at a higher concentration.

We have found substantial challenges appropriately controlling live vs. heat-killed experiments particularly with the specifics of our susceptibility experiments. With regards to the underlying question of mechanism we believe that the genetic mutants (e.g. rhlR/gacA) are equally informative and that further comparison of these mutants’ interaction with the C. elegans host as compared to wild-type may be informative.

> *(12) The authors should include a discussion on the relative virulence potential of PA01, PA14, and P. lurida and the relationship between bacterial virulence potential and the outcome of viral infection.*

We have also added data on mortality rates (Line numbers 183-200, Supplementary Figure 6). No significant mortality was observed within the 24-hour exposure period used for our Orsay infection and transmission assays. P. aeruginosa virulence is dependent upon temperature and as our assays are done at 20°C rather than 25°C this may account for reduced mortality compared to other published results. Regardless, we noted that O. vermis MYb71 killed C.

elegans as quickly as P. aeruginosa PA14 under these conditions and these two bacteria led to the shortest lifespan compared to the other tested bacteria. Interestingly, P. lurida MYb11 was observed to be more virulent than P. aeruginosa PA01 under these conditions. These results suggest that there is no direct correlation between mortality and susceptibility to Orsay virus, although it does not rule out that virulence effects unique to each bacterium could contribute to alterations in host susceptibility.

> *(13) More information is needed on strains listed in Supplementary Table 2, particularly when there is no reference listed and the strain is "Gift of XXX lab". For example, the Troemel lab previously published about an Ochrobactrum strain in Troemel et al PLOS Biology 2008 PMID: 19071962 - is this the same strain? Please ensure that there is adequate information about each strain with as many published references as possible so that the work can be more easily reproduced.*

We have added additional information and references to the strain table in Supplementary Table 2. The strain listed as Ochrobactrum sp. has been amended to Ochrobactrum BH3 as it is the strain described in Troemel et al. 2008.

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