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

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.

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 be observed spreading 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]. We set up a transmission assay by placing infected animals ("spreaders") together with uninfected animals on a monoaxenic lawn of each bacteria and examining the incidence proportion, or, 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** and **1B**). 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.

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

We reasoned that increased transmission of Orsay virus could be due to bacterial effects on the shedding of virus by infected spreader animals or bacterial modulation of host susceptibility to viral infection. 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 a fixed amount of Orsay virus to plates of each bacterial species and monitored infection using the *pals-5p::GFP* reporter. Specifically, we added Orsay virus in doses that ranged across four orders of magnitude and then quantified the fraction of individuals that became infected after 24 h (**Fig. 1A** and **1D**). 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**). 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**). We further quantified these impacts on host susceptibility by calculating the dose of Orsay virus required to infect 50% of the population after 24 h of exposure, which we defined as the ID_{50} (**Fig. 1D** and **1E**). On average we observed that the ID_{50} in the presence of *E. coli OP50* was 17-fold higher than the ID_{50} in the presence of *O. vermis* MYb71 (**Fig. 1E**). The ID_{50} observed in the presence of *P. lurida* was 120-fold and 2300-fold higher than the ID_{50} in the presence of *E. coli* OP50 or *O. vermis* MYb71 respectively (**Fig. 1E**).

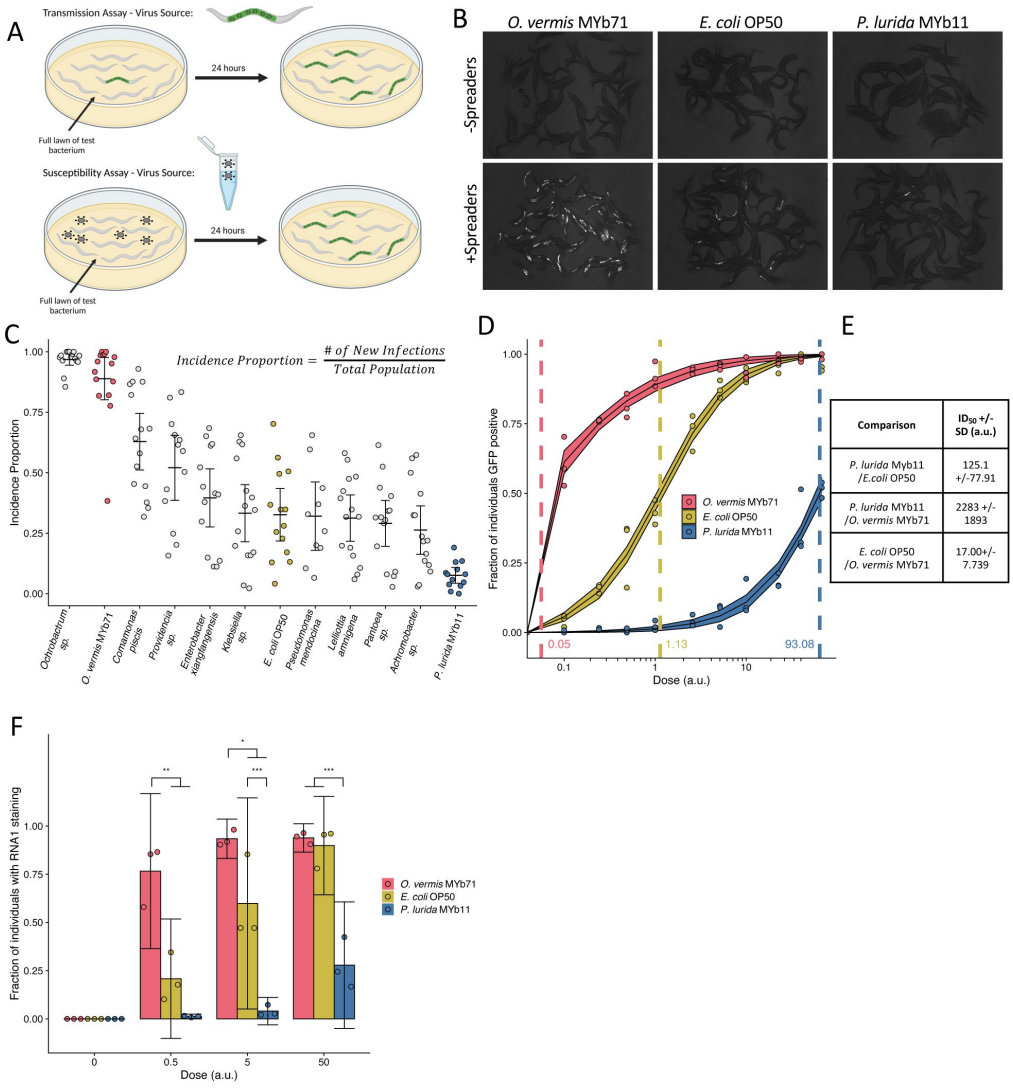


Figure 1

Ochrobactrum **species and** ***P. lurida*** **MYb11 divergently modulate Orsay virus transmission and infection rates.**

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

We corroborated our observations from the *pals-5p::GFP* reporter by scoring a susceptibility assay 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.

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[30,42]. 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**). Once more we observed substantial variation in the incidence proportion in the presence of *E. coli* OP50, while the incidence proportions in the presence of either *O. vermis* MYb71, *P. aeruginosa* PA01, or *P. aeruginosa* PA14 had minimal variation, suggesting a potent overall effect on transmission under these conditions (**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. 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**). 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 33% and 62% of the population respectively compared to *E. coli* OP50 which supported infection of 85% 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 the presence of *P. lurida and P. aeruginosa* species to sharply reduce and even effectively block, host susceptibility to infection with Orsay virus.

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

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[43]. 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[43]. 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

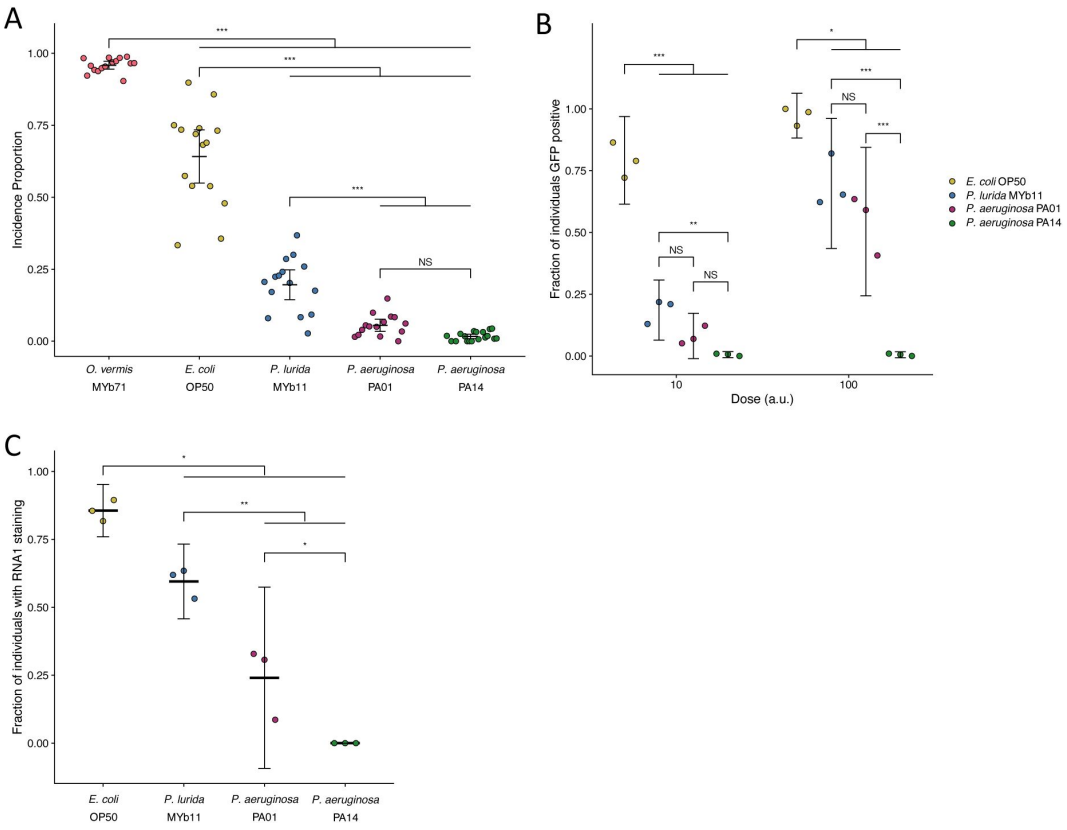


Figure 2

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

efficiency[43]. 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 following rare events of natural infection of *C. elegans* in the presence of *P. aeruginosa* PA14. We exposed young adult wild-type (N2) or RNAi defective animals (*rde-1(ne219)*) to exogenous Orsay virus while in the presence of *P. aeruginosa* PA14 and quantified the amount of virus present at 2 h and 24 h post-exposure, with the difference in RNA levels at these points reflecting viral genome replication. In the N2 background viral RNA1 levels increased by 3.5-fold at 24 h relative to levels at 2 h post-exposure (**Fig. 3B**). Orsay virus is more effective at replicating in the *rde-1* background compared to N2 and in the *rde-1* background viral RNA1 levels increased by 9000-fold at 24 h relative to levels at 2 h post-exposure (**Fig. 3B**)[23]. Together, these results suggest that Orsay virus RNA1 can also replicate once natural infection is achieved in both the wild-type and *rde-1*- defective background while animals are in the presence of *P. aeruginosa* PA14. These data confirm our findings that bacteria induced differences in viral replication do not explain the substantial attenuation of Orsay virus transmission in the presence of *P. aeruginosa* PA14.

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 behavior in response to growing population density[44]. *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**)[44]. 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**)[45–47]. *P. aeruginosa* possesses three exopolysaccharide biosynthetic clusters that each can 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[48–52]. 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**). Knockout of *gacA* in *P. aeruginosa* PA01 also suppressed the attenuation of Orsay virus infection (**Fig. 4B**). On the other hand, mutation of any of the three exopolysaccharide production pathways had no effect on infection (**Fig. 4B**). These data support a role for quorum sensing regulated processes in reducing Orsay virus infection rates but do not implicate a role for exopolysaccharide biosynthesis.

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**). Loss of the *rhlR* regulators *rhlI* or *pqsE* alone had no effect on the attenuation of Orsay virus infection, but

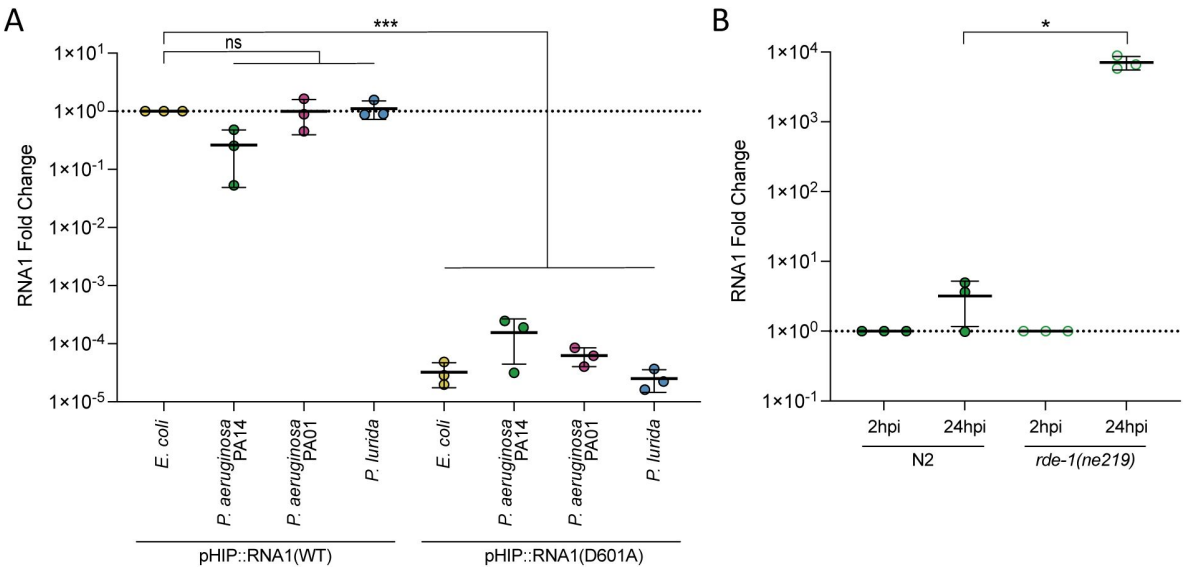


Figure 3

P. aeruginosa and *P. lurida* MYb11 do not eliminate Orsay virus replication.

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

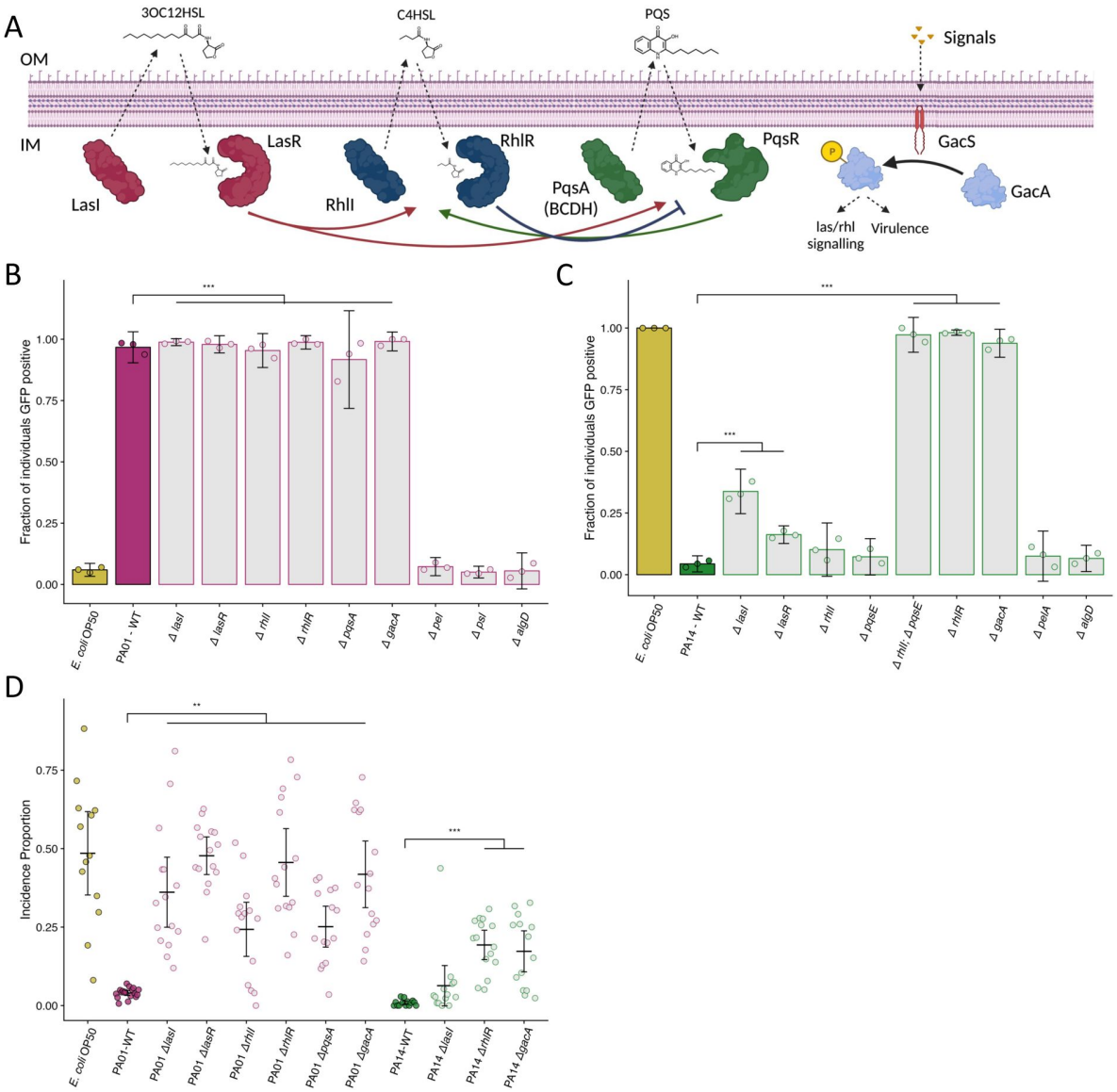


Figure 4

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

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

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**)[53]. Mutation of *lasI* or *lasR* suppressed the attenuation of Orsay virus infection to a lesser extent (**Fig. 4C**). Independent mutation of two genes responsible for *pel* or *alg* exopolysaccharide production had no effect on infection rates (**Fig. 4C**). 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 effects of wild-type *P. aeruginosa* on *C. elegans* infection in the presence of exogenous virus, we next confirmed that these *P. aeruginosa* mutants similarly affected not just host susceptibility, but also 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. 4D**). *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. 4D**). 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 specifically in *P. aeruginosa* PA14 to attenuate Orsay virus transmission.

It is additionally interesting to note that similar to the incidence proportion in the presence of *E. coli* OP50, the variation in the incidence proportion in the presence of all *P. aeruginosa* mutants increased (**Fig 4D**). This was true even for the incidence proportion observed in the presence of *P. aeruginosa* PA14 *lasI* mutant, which while not statistically different from wild-type *P. aeruginosa* PA14, had a qualitatively different distribution nonetheless (**Fig 4D**).

P. lurida 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*, a well-characterized pathogen of *C. elegans*, could inform us further regarding the mechanisms underlying the attenuation of Orsay virus transmission in the presence of *P. lurida* MYb11, a non-pathogenic constituent of the *C. elegans* microbiota[39]. In particular, we identified a *gacA* ortholog using Orthovenn2 and generated a putative knockout allele by removing the central 194 out of 214 amino acids (**Fig. 5A**)[54]. Following exposure to exogenous Orsay virus, knockout of *gacA* in *P. lurida* MYb11 led to infection of 80% of the population compared to infection of only 14% of the population in the presence of wild-type *P. lurida* MYb11 from exogenous Orsay virus (**Fig. 5B**). 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. 5C**). These data suggest that *gacA* has a conserved role across distant *Pseudomonas* species in the attenuation of Orsay virus transmission and infection of *C. elegans*.

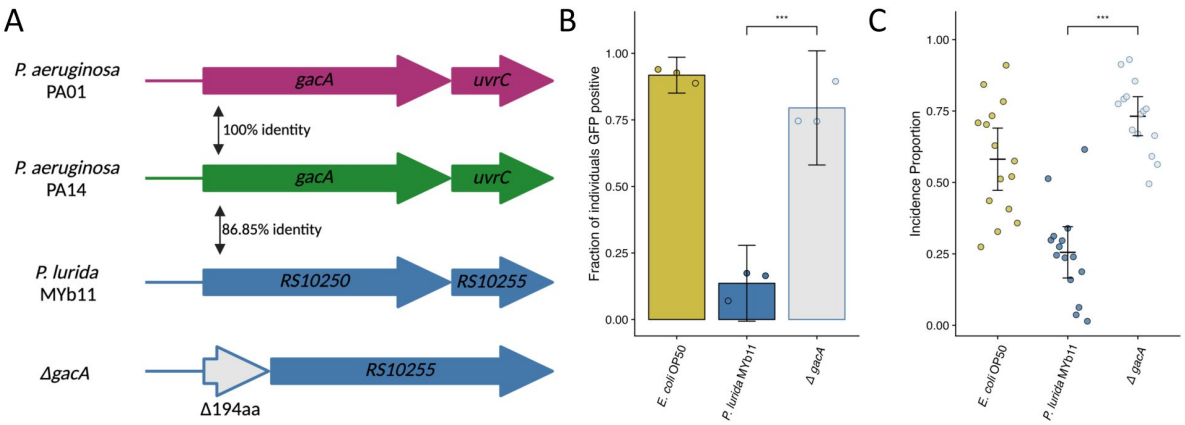


Figure 5

Mutation of *P. lurida* MYb11 *gacA* suppresses the attenuation of Orsay virus infection.

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

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. 6A**)[55]. 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 6A**)[47,56,57]. Of the 201 genes tested, 15 putative hits 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 these 15 candidate genes, we performed an additional set of susceptibility assays which confirmed six of the hits (**Fig. 6B**, 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 only 4.2% of the population in the presence of wild-type *P. aeruginosa* PA14. 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. 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 again 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. 6C**). However, similar to the effect observed with *lasI* mutation, the distributions of the incidence proportion observed in the presence of the *prpC*, *kinB*, or *glnK* mutants were qualitatively different from the nearly uniform incidence proportion observed in the presence of wild-type *P. aeruginosa* PA14 suggesting that mutation of these genes was impacting the attenuation of Orsay virus transmission to some extent (**Fig. 6C**).

All six of the hits originated from the set of genes required for full *P. aeruginosa* PA14 virulence in *C. elegans*. We noted that 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[57]. While these six genes have orthologs within *P. lurida* MYb11, 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(**Supp. Fig. 2A** and **2B**)[57,58]. One explanation of these results is that these genes play different roles within *P. lurida* MYb11 and therefore their mutation did not have the same consequences for Orsay virus attenuation. Alternatively, these results might further suggest that *ptsP*, *prpC*, and *kinB* act to attenuate Orsay virus infection via some specific effect on *P. aeruginosa* PA14 virulence.

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*. Moreover, we observe that pathogenic *P. aeruginosa*, which may be found in natural environments, can further attenuate Orsay virus

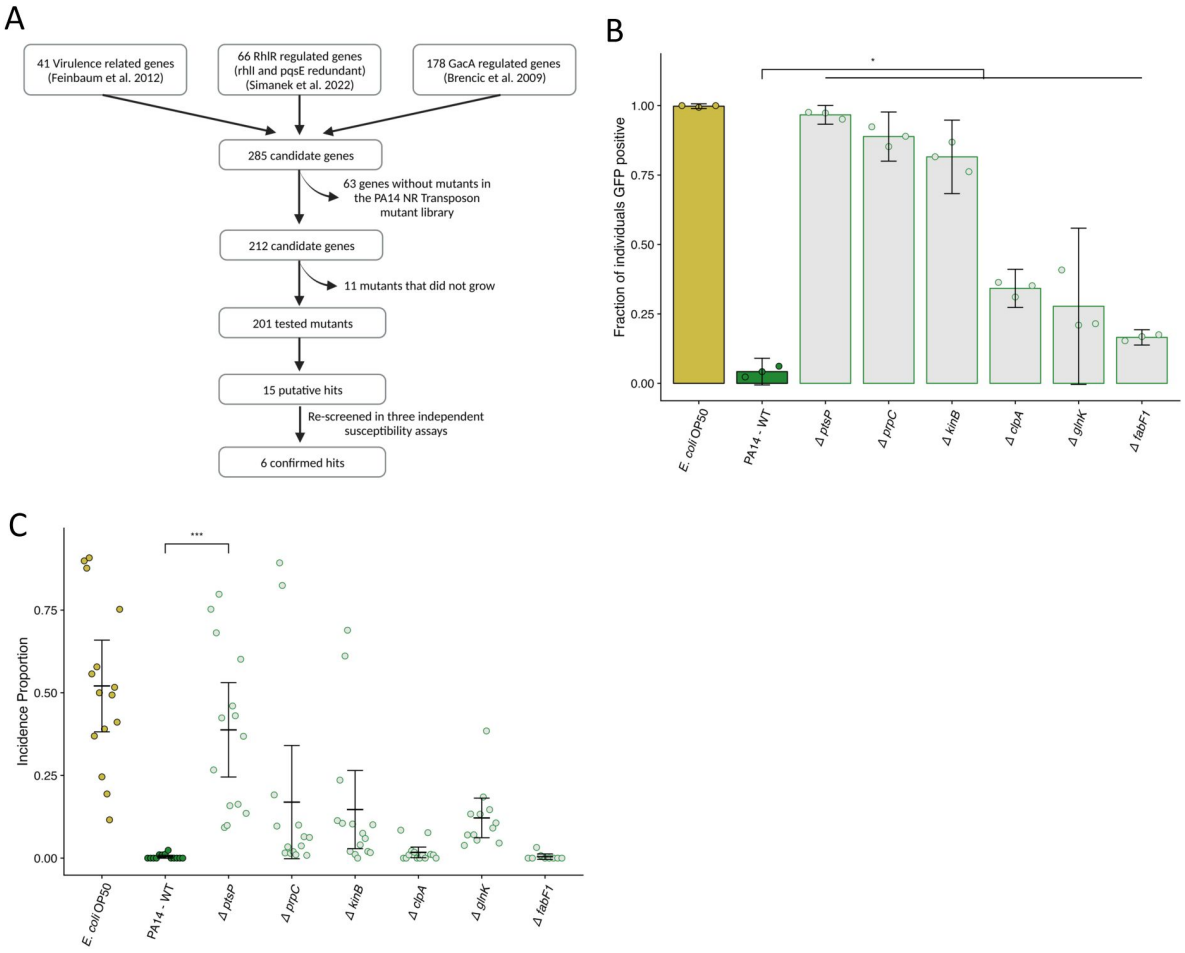


Figure 6

Mutation of *P. aeruginosa* PA14 genes related to virulence suppresses the attenuation of Orsay virus infection.

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

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[59].

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[60]. 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.

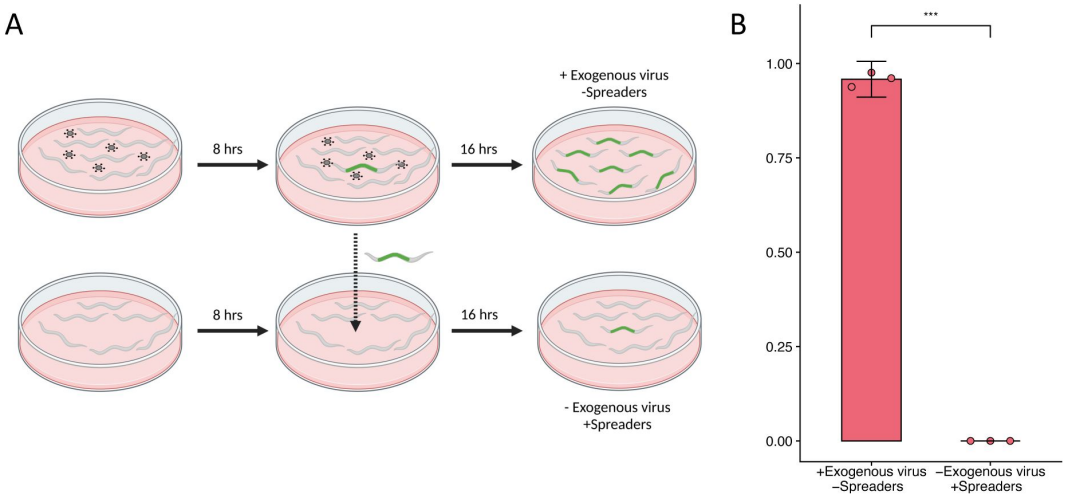
P. lurida MYb11 and *P. aeruginosa* PA01 and PA14 shared the ability to attenuate Orsay virus infection and transmission, even as *P. lurida* and *P. aeruginosa* have markedly distinct effects on the *C. elegans* host. *P. lurida* MYb11 promotes developmental rate and fitness, although may do so at a minor cost to overall lifespan[39,61]. In contrast, *P. aeruginosa* is a pathogen of *C. elegans* and numerous other organisms[30,62]. We identified that *gacA* regulates the attenuation of Orsay virus by *P. aeruginosa* PA01 and PA14 and *P. lurida* MYb11. The *gacS/gacA* system regulates many phenotypes in a variety of ψ-proteobacteria[63]. In Pseudomonads, *gacA/gacS* regulate processes related to quorum sensing, virulence, and biocontrol in a population density dependent manner[63]. These data raise the possibility that *Pseudomonas* quorum sensing pathways are required for the effects on Orsay virus transmission and infection. Quorum sensing also influences additional aspects of *P. aeruginosa* physiology, including swarming behaviors, biofilm formation, secondary metabolism rates, and overall transcription patterns[44].To gain additional insights into the *P. aeruginosa* effects on Orsay virus transmission we conducted a candidate-based screen including genes influenced by quorum sensing and genes that regulate virulence towards *C. elegans*[47,56,57]. We identified six genes, *ptsP*, *prpC*, *kinB*, *clpA*, *glnK*, and *fabF1*, that when mutated suppressed *P. aeruginosa* PA14 attenuation of Orsay virus infection. Each of these genes has been shown to be required for full *P. aeruginosa* PA14 virulence in *C. elegans*[57]. 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 against a role for general virulence in the attenuation of Orsay virus transmission and infection caused by *P. aeruginosa* PA14 and *P. aeruginosa* PA01[57].

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[64]. 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

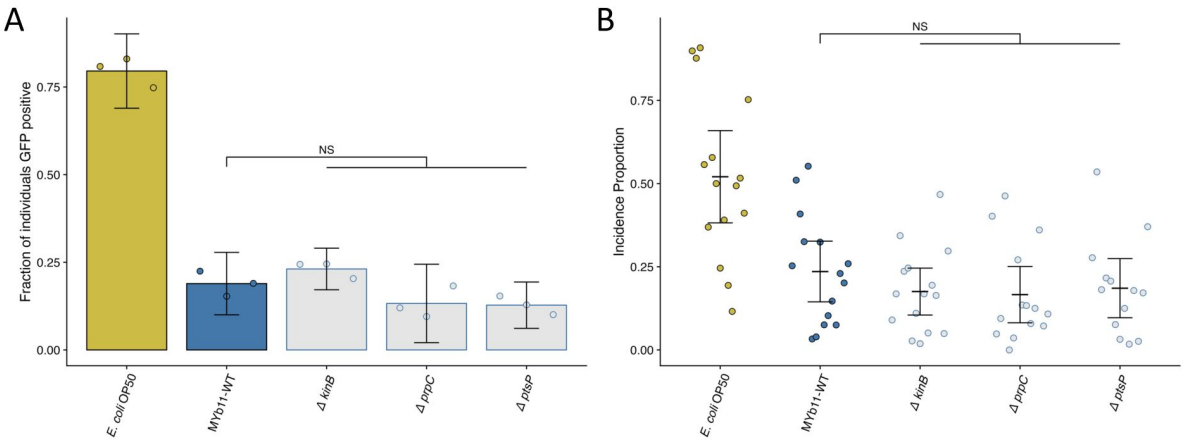
C. elegans were maintained on NGM agar plates (17 g agar, 2.5 g peptone, 3 g NaCl per 1 L water) containing *E. coli* OP50[41]. Strains bearing the *glp-4(bn2)* temperature sensitive mutation were maintained at 16°C, while all other were maintained at 20°C. All assays were performed on SKA



Supplementary Figure 1

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

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



Supplementary Figure 2

Knockout of the *P. lurida* MYb11 orthologs of *ptsP*, *prpC*, or *kinB* does not suppress Orsay virus attenuation in susceptibility or transmission assays.

(A) The fraction of individuals GFP positive following exposure to 10 a.u. of exogenous Orsay virus in the presence of the indicated bacteria. Data are from a single representative experiment. (C) Incidence proportion of Orsay virus transmission in the presence of the indicated bacteria. The *E. coli* OP50 reference is shared with **Figure 5C**. Data are from three experiments and dots represent individual plates. For all plots the black bar is the mean and error bars are the 95% confidence interval (C.I.). p-values determined using one-way ANOVA followed by Dunnett's test (NS non-significant, *p<0.05, **p<0.01, ***p<0.001).

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 dose testing

Orsay virus was isolated from infected WUM31(*rde-1(ne219);jyIs9[pals-5p::gfp;myo-2p::mCherryI*) 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 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, 3.5 cm SKA plates containing infected WUM31 animals was allowed to starve and then equally chunked onto four 10 cm NGM plates with *E coli* OP50. Once these plates were just starved, eight 10 cm plates were washed and homogenized as above. The homogenized suspension was then centrifuged at 13.2xg 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 *E. coli* OP50. Doses referenced throughout the manuscript refer to actual microliters applied to each plate. However, no method was used to normalize doses between batches of virus and so we have chosen to refer to doses in terms of arbitrary units (a.u.). The average ID_{50} while on *E. coli* OP50 for all virus batches used in this study was 3.6 suggesting an average dose of 1 a.u. roughly corresponds to 0.28x the ID_{50} on *Escherichia coli*.

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::mCherryI*) 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. 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 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. 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, 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 GFP positive individuals after 24 h was less than the initial number of spreaders placed on the plate were excluded.

Susceptibility assays

Unless otherwise indicated, Orsay virus filtrate at the indicated doses was diluted in filtered M9 solution. 200 uL 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 GFP-positive individuals was calculated.

Early Transmission Assay

For early transmission assay, young ZD2611 adults were exposed to 0a.u. or 5a.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.

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.

RNA Extraction

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

qPCR

cDNA was made using 500ng of RNA as template (Promega GoScript Reverse Transcriptase/Random Primers). cDNA was diluted at 1/40 and qPCR were performed using 1ul 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].

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[43]. 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. aeruginosa* PA14

Adult ERT54 (*jyIs8[pals-5p::gfp;myo-2p::mCherry])* or WUM31 (*rde-1(ne219); jyIs8[pals-5p::gfp;myo-2p::mCherry)]* were exposed to exogenous Orsay virus in the presence of *P. aeruginosa* PA14. 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.

Pseudomonas lurida MYb11 mutant construction

A protocol developed for allelic exchange in *P. aeruginosa* was modified for use in *P. lurida* MYb11[65]. 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)[66]. 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[65]. Colonies that survived were genotyped for the expected deletion.

Data visualization and statistics

All experiments were performed three times. For transmission, qPCR, and FISH-based susceptibility assays data from each experiment are combined. For susceptibility assays a single representative experiment is shown. Data were analyzed in R Studio[67]. 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 explicitely noted. Susceptibility assay curves were modeled using the drc[68] 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. Plots were made using the gdata[69], scales[70], drc[68], Rmisc[71], multcomp[72], ggplot2[73], ggsignif[74], and cowplot[75] packages.

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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.

I was also curious why the authors did not further explore the mechanism behind the quorum-sensing 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 OOP50, Pseudomonas, or the quorum sensing knockout of Pseudomonas? That would reveal whether it is the compound itself vs. something that the compound does.

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?

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.

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.

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.

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.

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.