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Comparative pathology of bacteria in the genus *Providencia* to a natural host, *Drosophila melanogaster*

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Abstract

Bacteria in the genus *Providencia* are pathogens of many organisms, including humans and insects. We and colleagues have isolated five different strains belonging to four distinct *Providencia* species as natural infections of *Drosophila melanogaster* captured in the wild. We found that these isolates vary considerably in pathology to infected *D. melanogaster*, differing in the level of mortality they cause, their ability to replicate within the host and the level that the fly's immune response is elicited. One interesting bacterium was *Providencia sneebia*, which causes nearly complete mortality and reaches large numbers in the fly but does not elicit a comparably strong immune response. Through coinfection experiments, we determined that *P. sneebia* avoids recognition by the immune system. We tested for biofilm formation and replication within *D. melanogaster* cells as possible mechanisms for *P. sneebia* escape from host immunity, but did not find evidence for either. *D. melanogaster* and *Providencia* provide a powerful system for studying general host-pathogen interactions, and for understanding how the well-studied immune model host *D. melanogaster* interacts with its natural bacterial pathogens.

Keywords

Providencia; *Drosophila melanogaster*; Pathogens; Host-Pathogen Interactions; Virulence; Innate Immunity

1. Introduction

Closely related bacterial pathogens may utilize a wide range of mechanisms to infect hosts, in part because virulence mechanisms are genetically labile and are often horizontally transferred between reasonably distantly related microbes [1]. Understanding differences in pathology between closely related bacteria highlights recent shifts in virulence, and can ultimately lead to the identification of the underlying genetic basis. Several strains and species of the γ-Proteobacterial genus *Providencia* have recently been isolated from field infections of wild caught *Drosophila melanogaster* [2, 3, P. Juneja and S. M. Short unpublished], and in the present work we contrast the pathological interactions of these bacterial species within their *Drosophila* host. *D. melanogaster* is a well established model host for studying innate immunity [4] and the pathology of virulent bacteria [e.g. 5–8], but

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few natural pathogens of *D. melanogaster* have been identified or extensively studied [but see 9–11]. We believe that *Drosophila* and *Providencia* comprise a powerful natural system for the study of variation in virulence and host-pathogen interactions. Because many microbial virulence strategies focus on conserved aspects of eukaryotic physiology and cell biology, inferences from this system can apply across broad host ranges, including from insects to humans.

Bacteria of the genus *Providencia* are Gram-negative opportunistic pathogens that have been isolated from a wide variety of environments and organisms ranging from humans to insects to sea turtles and shark mouths [12–15]. *Providencia rettgeri*, *Providencia alcalifaciens*, and *Providencia stuartii* have all been isolated from human stool samples both as part of the natural human gut flora and as the cause of gastric upset such as traveler's diarrhea [16–18]. Some strains of *P. alcalifaciens*, but no strains of *P. rettgeri* or *P. stuartii*, have been found to be intracellularly invasive in human cell lines [16, 17, 19, 20]. *Providencia* also cause urinary tract and other nosocomial infections in humans [12, 13]. Numerous studies surveying bacteria associated with insects such as blowflies, stable flies and Mexican fruit flies have isolated *Providencia* species either from the whole insect or specifically from the gut [e.g. 21–23], although it is unclear whether these and other associations have meant the bacteria were acting as pathogens or were simply present in the insects' environment. *Providencia* have been recurrently found in association with *D. melanogaster*, including in a survey for bacterial associates in a natural population [24], in the hemolymph of laboratory cultures of *domino* mutant larvae that are void of hemocytes and generally sick with bacterial infections [25], and as natural infections in wild-caught *D. melanogaster* [2, 3, P. Juneja and S. M. Short unpublished].

The *D. melanogaster* innate immune system has been well described, primarily from experiments measuring the response to injection of avirulent bacteria or generic immune elicitors [reviewed in 4]. *D. melanogaster* is also an excellent model for studying the pathology of virulent bacteria, since many virulence mechanisms are effective across a broad range of hosts. As a result, *Drosophila* has successfully been used as an experimental host to model clinical pathogenesis in humans and animals, insect vectoring of human disease and microbiological control of insect agricultural and medical pests. For example, *Drosophila* have been used to study opportunistic human infectors such as *Serratia marcescens* and the pathologies of *Pseudomonas aeruginosa* communities found in cystic fibrosis patients [7, 26]. *D. melanogaster* has also stood in as a model host for other arthropods such as ticks and mosquitoes that bear *Francisella tularensis* infections, ticks that host *Ehrlichia chaffeensis*, and caterpillars infected with *Photorhabdus luminescens* vectored by entomopathogenic nematodes [27–29].

Despite previous studies of bacterial pathogens of other animals using *D. melanogaster* as a model host, very little is known about the bacteria that infect *D. melanogaster* itself in its natural habitat. In some of the few efforts to identify bacterial pathogens of wild *Drosophila*, four different species belonging to the genus *Providencia* were recovered along with isolates of other bacteria from the hemolymph of wild caught *D. melanogaster* [2, 3, P. Juneja and S. M. Short unpublished]. Since the hemolymph of a healthy fly should be sterile, the presence of bacteria can be considered to constitute an infection. Two of the recovered *Providencia* species are the previously described *Providencia rettgeri* and *Providencia alcalifaciens* [12]. The other two species were identified as novel species named *Providencia sneebia* and *Providencia burhodogranariea*, the latter of which has two distinct strains designated B and D [3].

In this paper, we determine the pathology of *Providencia* species and strains in *D. melanogaster*, where pathology is defined as the proportion of mortality caused by the

bacteria, the bacterial ability to proliferate within the fly, and the levels of host immunity induced by infection as measured by the expression of antimicrobial peptide (AMP) genes. We find *Providencia* to be highly variable in all three phenotypes. The ability of the bacteria to proliferate within the fly, the amount of AMP expression, and the level of mortality the bacterial cause are often all positively correlated, with the most deadly bacteria reaching the highest amount within the fly and inducing the highest levels of AMP expression. A notable and interesting variation to this pattern is *P. sneebia*, which kills about 90% of infected flies and reaches very large numbers in these flies but induces less AMP expression than other *Providencia* species, even those that cause significantly lower mortality and do not proliferate as effectively within flies. Through coinfections with *P. sneebia* and *P. rettgeri*, we concluded that *P. sneebia* is able to actively avoid recognition by the fly's immune system and is resistant to ectopic immune induction. Two possible hypotheses to explain these observations are that *P. sneebia* invades and replicates within insect cells or forms a biofilm during infection, but we do not find evidence supporting either hypotheses *in vitro*, suggesting that *P. sneebia* virulence mechanisms are more complicated. The diversity of virulence profiles we observe among these *Providencia* isolates indicates they will be a rich substrate for future study of *Providencia* infection dynamics in a natural and experimentally tractable host.

2. Methods and materials

2.1. Fly stocks and bacteria strains

D. melanogaster fly stocks that were used were either wild type Oregon R (OreR), OR;imd^{10191};OR [30], Toll 1-^{RxA},ry,h,st,e/Tm3 Ser [31], or expressing green fluorescent protein (GFP) under the promoter of the AMP *Diptericin A* (*DptA*), *DptA*-GFP [32]. They were maintained on standard glucose medium (12 g agar, 100 g glucose and 100 g Brewer's yeast per 1.2 L of water, plus a final concentration of 0.04% phosphoric acid and 0.4% propionic acid added to inhibit microbial growth in the food) and kept at room temperature (22–24 °C). Table 1 provides a complete list of *Providencia* bacterial strains. All *Providencia* strains were grown in LB media at 37 °C overnight with shaking, except for *P. burhodogranariea* strains, which were grown at 25 °C. *Listeria monocytogenes* 10403S was grown at 37 °C in BHI medium with shaking. *E. coli* Mach1-T1, a cloning strain (Invitrogen Corp), was grown at 37 °C in LB medium with shaking.

2.2. Mortality

Overnight cultures used for infecting flies were grown to saturation and then diluted to an A_{600nm} of 1.0. To deliver infections, a 0.15 mm minuten pin (Fine Science Tools) mounted on a 200 μL pipet tip was dipped into the diluted overnight culture and poked into the thorax of a CO_2 anesthetized fly. This delivers about 10^3 to 10^4 bacteria to each fly. Sterilely wounded flies were pricked with a needle that was sterilized in 95% ethanol. Anesthetized control flies were handled in the same way as the others but were not wounded. Flies were maintained in vials with food at room temperature and surviving flies were counted once a day for 6 days after infection. Infection with each bacterium was performed on at least 2 days with controls done on each day. Product limit survival estimates and homogeneity by log-rank tests were conducted using proc lifetest in SAS version 9.1 (SAS Institute). P-values were corrected for multiple tests in some cases by a Bonferroni correction with a cut off value of p=0.0025 for comparing all strains that are the focus of the paper, p=0.00625 for comparing among *P. sneebia* isolates only, and p= 0.025 for comparing only among *P. burhodogranariea* strains. In contrasts of different strains of *P. burhodogranariea*, only those infections that were preformed on the same day were compared.

2.3. Bacterial load

To measure systemic bacterial load in infected flies, single OreR flies were infected by pinprick as described in section 2.2, then homogenized in 500 μL LB and plated by robotic spiral platers (manufactured by Don Whitley Scientific and Spiral Biotech) on LB agar plates at 2, 4, 6, 10, 18, 24, and 32 hours post infection. Flies were kept in vials with food at room temperature between infection and homogenization. The LB agar plates were incubated overnight at 25 °C for *P. burhodogranariea* or 37 °C for *P. rettgeri*, *P. alcalifaciens*, *P. sneebia* and sterile wound. Gut commensal bacteria grow more slowly than *Providencia* under these conditions, so by limiting incubation to overnight we exclude any commensal bacteria from our assay. The number of colony forming units (CFU) on each plate was recorded using a counter associated with the spiral platers, allowing the concentration of viable bacteria in each homogenate to be calculated based on the number and position of colonies on the plates. Bacterial loads for flies infected with *P. alcalifaciens* and *P. sneebia* were compared at each individual time point using proc glm in SAS version 9.1 with the model: ln(CFU+1)=bacterial treatment + sex. The boxplot was generated using the function boxplot in R. A small number of surviving flies from each treatment were also homogenized at 7–10 days post infection as described above.

2.4. Antimicrobial peptide expression

We first examined *DptA*-GFP flies to determine how much AMP expression occurred during infection. We infected flies on replicate days as described in section 2.2 and kept them in vials with food until the time examined. Other AMP promoters examined which had undetectable levels of fluorescence were *Defensin*, *Drosocin*, *Attacin* and *Cecropin* [32]. At 6, 24, and 32 hours post infection flies were anesthetized and examined under a dissecting scope and scored for the intensity of GFP fluorescence blind of the treatment. This assay was restricted to females because males were found to have too much background fluorescence.

For quantification of AMP expression by QPCR, OreR flies were either infected with a bacterium or sterilely wounded as described in section 2.2 then were frozen at −80 °C in pools of 8 flies at 0, 2, 4, 6, 10, 18, 24, and 32 hours post treatment. Flies were maintained in vials with food at room temperature between infection and freezing. Each treatment was performed on at least two different days. Total RNA was extracted with Trizol (Invitrogen Corp) using the manufacturer's suggested protocol, then reverse transcribed to cDNA from poly-T primers using standard procedures. The abundances of the AMPs *Diptericin A* (*DptA*), *Drosomycin (Drs*) and *Defensin* (*Def*) and the housekeeping gene *rp49* were quantified by QPCR on an ABI 7000 Sequence Detection System (Applied Biosystems) using specific TaqMan primers and the manufacturer's suggested protocol (primer and probe sequences available upon request). For statistical analysis, gene expression at each hour was examined separately in proc glm in SAS version 9.1 using the model: AMP Ct = Rp49 Ct + treatment + date infected. Correction for multiple tests was achieved using the Tukey-Kramer method. Least squares means were recovered at the mean Rp49 Ct. Fold induction was calculated as 2 to the power of the difference between the Ct of the sterile wound control and the Ct of the infection treatment for each time post-infection.

2.5. Coinfection

For coinfections, overnight cultures of *P. rettgeri* and *P. sneebia* were grown to saturation and then diluted to an A_{600nm} of 2.0. The bacteria were then mixed at proportions 1:1, 1:3 or 3:1 with either the alternate bacteria or LB. Flies were then infected in the thorax with a small needle dipped in the culture as described in section 2.2, replicated on two different days. Although three different proportions of each bacterium were examined, we found that the results were the same for each infection class (singly infected *P. rettgeri*, singly infected

P. sneebia, or coinfected) regardless of the mixing proportion, allowing us to pool all proportions in final analyses. We only examined male flies for AMP expression and bacterial load in the coinfection because we had found no difference between the sexes in our primary examination of mono-infections. At 6, 24, and 32 hours post infection flies were frozen at −80 °C. RNA extraction, QPCRs, and statistical analysis for AMP expression were performed as described in section 2.4. Fisher's combined probability was used to summarize the independent expression experiments.

For the examination of AMP expression in *DptA*-GFP flies, infected or control flies were placed in vials with standard fly food and examined blind of treatment at 6, 24, and 32 hours post infection with a dissecting scope. Here, only female flies were examined due to male background fluorescence. The survival of these same flies was monitored up to six days post infection and statistically analyzed as in section 2.2.

Determination of the bacterial load of coinfected flies and statistical analysis was carried out as described in section 2.3. To distinguish between *P. sneebia* and *P. rettgeri*, we took advantage of *P. rettgeri*'s natural resistance to tetracycline. All samples were plated on LB plates without antibiotic and on plates with a tetracycline concentration of 10 μg/mL. The number of CFU on the tetracycline plates was inferred to be the count of *P. rettgeri* and the difference in CFU between the paired plates was assumed to be the *P. sneebia* count. PCR and restriction enzyme digestion of the 16S gene looking for species-specific digestion pattern was done to check that the proper species were growing on the correct plates. This experiment was carried out twice on different days.

2.6. Biofilm formation

Overnight bacteria cultures were diluted to an A_{600} of 1.0, then gently centrifuged into a pellet and washed three times with 1X PBS, and ultimately concentrated to 20X. 5 μl of bacteria or PBS, as a control, were added to 200 μL of Schneider's media with 10% fetal calf serum in a 96-well plate. Bacteria that received the antibiotic treatment sat in media alone for approximately 1 hour before the antibiotics were added to the well. The antibiotics ceftazidime and kanamycin were added to a final concentration of 1mg/mL and 200 μg/mL, respectively. At 6 and 24 hours after the bacteria or antibiotics were added to the media, the wells were washed three times with sterile water before the addition of 0.1% crystal violet, which was incubated for 15 minutes. The wells were then washed twice with water before drying for 5 hours. 30% acetic acid was added to the wells to solubilize the crystal violet. The A_{540nm} was read using Multiskan Spectrum plate reader (Thermo Scientific). The final absorbance was calculated as the difference from the PBS control well at that time.

2.7. Antibiotic protection assay

D. melanogaster S2 cells were maintained in Schneider's media with 10% fetal calf serum at 25 °C. For the antibiotic protection assay, cells were seeded in 6 well plates the day before the assay was carried out so that there would be approximately 10^5 cells/mL the next day. Overnight cultures of bacteria were washed three times with PBS before addition to the wells containing S2 cells at a multiplicity of infection of 10. After two hours the media was removed and the cells, which lightly adhere to the bottom of the wells, were washed while still in the wells three times with PBS. Schneider's media with 10% fetal calf serum containing 1 mg/mL ceftazidime and 20 μg/mL kanamycin was then added to the wells. Neither ceftazidime nor kanamycin should penetrate eukaryotic cell membranes, so only extracellular bacteria should be killed. The cells were incubated with the antibiotics for 2 hours to kill extracellular bacteria. At 0, 6 and 24 hours following this two-hour incubation, the media only was removed from the wells and centrifuged. The pellet was then washed with water before being plated on BHI or LB plates, depending on the bacteria, to provide

an estimate of the number of viable bacteria in suspension (this number should be near zero because of the presence of antibiotics). The S2 cells were then washed off with water and spun down and washed again with water. The pellet was then resuspended in BHI media before being plated on either BHI or LB. CFUs were manually counted to yield the number of viable bacteria residing inside the S2 cells.

3. Results

3.1. Mortality

Given that closely related bacteria often vary in their virulence to a given host, we hypothesized that the different strains of *Providencia* isolated from wild caught *Drosophila melanogaster* might also vary in pathology (Table 1). There was minimal mortality (5–10%) among control flies either only anesthetized on CO_2 or wounded with a sterile needle. When flies are infected with *P. burhodogranariea* strain D, less than 10% of infected flies died by six days post infection (Fig. 1A). This is not significantly different from the amount of mortality from either control (Fig. 1A; *P. burhodogranariea* strain D contrasted to CO_2 control: p=0.0612, *P. burhodogranariea* strain D-sterile wound: p= 0.0436, not significant after correcting for multiple tests). About 40% of flies infected with *P. burhodogranariea* strain B die from the infection, which is highly significantly different from *P. burhodogranariea* strain D ($p < 0.0001$), although for unknown reasons, *P. burhodogranariea* strain B infections displayed more day-to-day variation in mortality than infections with any other bacteria with mortality rates ranging from 20% to 60%. *P. rettgeri* strain Dmel likewise caused moderate mortality, with fewer than 50% of the flies dying. The amounts of mortality caused by *P. rettgeri* and *P. burhodogranariea* strain B are not significantly different from each other (p=0.0303), although both infections caused significantly higher mortality than is observed in controls (all $p < 0.0001$). *P. sneebia* strain Type and *P. alcalifaciens* strain Dmel each caused much greater mortality than any of the other species. Both within the first two days of infection, *P. sneebia* kills about 90% of infected flies and *P. alcalifaciens* causes mortality in 99% of infected flies. Mortality from infections with each *P. sneebia* and *P. alcalifaciens* is significantly different from all other treatments, including each other (all $p < 0.0001$). Thus, there are three major classes of virulence among our isolated *Providencia* as defined by mortality: *P. burhodogranariea* strain D causes minimal mortality, *P. rettgeri* and *P. burhodogranariea* strain B cause moderate amounts of mortality, and *P. sneebia* and *P. alcalifaciens* are highly virulent.

Multiple isolates of *P. sneebia* and *P. burhodogranariea* strain B have been recovered from the hemolymph of wild caught *D. melanogaster* (Table 1) [2, 3]. We infected flies with each of these to test whether there is heterogeneity among isolates in the mortality caused by these strains. Of the two other isolates of *P. burhodogranariea* strain B, only isolate B97 is significantly different than the Type strain B, with B97 causing less mortality (Suppl. Fig. 1; Suppl. Table 1; $p = 0.0003$). Eight *P. sneebia* isolates were tested and all caused greater than 80% mortality, although some of them cause slightly but significantly different mortality than the Type strain (Suppl. Table 1). This suggests that while there is some variation among isolates, *P. sneebia* can be considered to always be highly virulent while *P. burhodogranariea* is never highly virulent.

The Drosophila humoral immune response is activated by two major signaling pathways, the Toll pathway and the Imd pathway [4]. The Imd pathway tends to be more responsive to Gram-negative bacteria, whereas the Toll pathway preferentially activated by Gram-positive bacteria. We therefore hypothesized that the Imd pathway would be most important in fighting *Providencia*. We measured the mortality of flies that were mutationally deficient in either the Toll or Imd pathway after infection with *Providencia*. We found Toll pathway mutants showed no significant difference in mortality compared to wild type flies after

infection with either strain of *P. burhodogranariea* or with *P. rettgeri* ($p>0.05$, in all cases). *P. sneebia* and *P. alcalifaciens* did cause significantly greater mortality in the Toll mutant flies compared to the wild type flies ($p<0.05$, in both cases), but flies of both genotypes suffered severe mortality within 2 days of infection with these bacteria (Suppl. Fig. 2A). In contrast, Imd mutant flies infected with any strain of *Providencia* suffered very high mortality within 2 days post infection (Suppl. Fig. 2B). Notably, we observed high mortality in flies infected with the *P. rettgeri* and *P. burhodogranariea* strains, which cause only moderate to low mortality in wild type flies (Suppl. Fig. 2B). All *Providencia* infections in Imd mutant flies were significantly different than those seen in infected wild type flies ($p<0.05$, in all cases). These data indicate that the Imd pathway is essential to fighting *Providencia* infection, and that *P. rettgeri* and *P. burhodogranariea* infections are controlled by the host immune system and not simply limited by inherent failure of the bacteria to be able to colonize the fly.

We were intrigued by the recurrent isolation of diverse *Providencia* species from *Drosophila*, so we examined the amount of mortality caused in *D. melanogaster* by the Type strains of 6 *Providencia* species isolated in other contexts, including *P. rettgeri* and *P. alcalifaciens* isolates not derived from *Drosophila* (Table 1; Suppl. Fig. 3). Except for *P. alcalifaciens* strain Type, all species caused less than 20% fly mortality in wild type flies. The Type strain of *P. alcalifaciens* caused less mortality than our Dmel strain (Suppl. Fig. 2; $p<0.0001$), which suggests there are genetic differences between the strains. The Type strain of *P. rettgeri* also caused less mortality than our Dmel strain (Suppl. Fig. 2; $p<0.0001$). These data indicate that the high amount of *Providencia* induced *D. melanogaster* mortality is specific to those strains that were isolated from wild flies.

3.2. Bacterial load

For a given host and pathogen pair, bacterial proliferation and host mortality may or may not be correlated. To test our hypothesis that the *Providencia* species that cause the highest mortality are those that are best able to replicate in flies, we measured the number of bacteria present in *D. melanogaster* at multiple time points for the first 32 hours after infection. Plates from control flies that were sham-infected with a sterile needle did not have any bacteria growth after the overnight incubation period (data not shown), indicating that the control flies did not have any *Providencia* within or on them. Commensal bacteria from the gut begin to appear on all plates after they have been incubated for at least 24 hours. Infections with the five bacteria start to diverge in CFU counts around 10 hours post infection (Fig. 1B). There are a few individual flies that are able to clear the infection during the first few hours. It is unclear why some flies are able to clear their infections and others are not, although we suspect it reflects minor heterogeneities in the infection process.

Flies infected with either strain of *P. burhodogranariea* cleared their infections or maintained stable bacterial loads around the level of the initial introduction over the first 32 hours of infection (Fig. 1B). These bacteria are eventually cleared from all surviving flies, as survivors have no bacteria present 7–10 days post infection (data not shown). *P. rettgeri*, *P. sneebia* and *P. alcalifaciens* all show an increase in the number of CFU per fly after 6 hours of infection. Among the flies infected with *P. rettgeri*, there is a large amount of variation in the number of bacteria present in individual flies at 24 and 32 hours post infection, ranging from 10^3 to 10^7 CFU per fly (Fig. 1B). It seems likely that this variation reflects divergence in the infection trajectory among individual flies, where those with the highest bacterial loads probably succumb to the infection and the others survive. Flies that survived their infections for 7–10 days post infection carried either no CFU or between 10^2 and 3×10^4 CFU per fly (data not shown). Both *P. sneebia* and *P. alcalifaciens* are able to rapidly proliferate to very high numbers in the fly by 32 hours post infection, which is shortly before flies die from these infections. The number of bacteria present in the infected flies is

significantly different between *P. sneebia* and *P. alcalifaciens* at 18 hours post infection ($p=0.0115$), but not at 24 and 32 hours (both $p>0.05$). Approximately 10% of the total *P. sneebia* infected flies had no bacteria present at their time of sampling. These flies were most likely able to clear the bacteria within the first few hours of infection and probably represent the small percent of flies that survive in the mortality assays (Fig. 1A). This hypothesis is supported by the observation that flies infected with *P. sneebia* that survive 7–10 days post infection are free of *Providencia* (data not shown). In total, across all species, these data demonstrate that the *Providencia* species that are best able to proliferate within the fly are those that cause the highest mortality.

3.3. *D. melanogaster* immune response to infection

Insects respond to the presence of bacteria by activating their humoral immune system, which results in the production of antimicrobial peptides (AMPs). Induction of AMP gene expression varies among different microbes and immune elicitors [33], and we hypothesized that the *Providencia* bacteria that were most proliferative during infection would cause the highest induction of the immune response. To initially test this hypothesis, we infected transgenic flies that express GFP driven by AMP promoters [32] then examined individual flies by eye at 6, 24, and 32 hours post infection. Although the expression patterns of several different AMPs were examined (see section 2.4), only *DptA* produced a strong fluorescence after infection with most *Providencia*. Both *P. burhodogranariea* strains failed to drive detectable fluorescence signal even with *DptA*. As expected, *DptA*-GFP expression was localized to the immune responsive fat body. We repeatedly saw that flies that were infected with *P. sneebia* showed lower fluorescence than flies infected with *P. alcalifaciens* or *P. rettgeri*, both in the intensity of the GFP expression as well as the proportion of a single fly expressing GFP (Fig. 2A). This contrasted with our expectation based on the high levels of *P. sneebia* proliferation within flies and host mortality caused from infection.

We used QPCR of AMP mRNAs to better quantify the immune response of infected flies for the first 32 hours of infection relative to control flies that were wounded with a sterile needle. By calculating the fold induction over the sterile wound, we could determine the amount of AMP expression that was specifically attributable to the bacteria and not to the wound in delivering the infection (Fig. 2B). Consistent with our observations of the *DptA*-GFP flies, *P. sneebia* infections consistently resulted in lower expression of *DptA* than *P. rettgeri* and *P. alcalifaciens* did at later times in the infection progression. At 24 and 32 hours after infection with *P. sneebia*, *DptA* expression was not significantly different from expression in response to the sterile wound alone (Fig. 2B; both $p>0.05$). In contrast, *P. rettgeri* and *P. alcalifaciens* induced significantly higher levels of expression than the sterile wound at 24 and 32 hours after infection (Fig. 2B; in all cases $p<0.05$). None of the bacterial infections drove *DptA* expression above the level seen from sterile wound alone prior to 24 hours post infection, and flies infected with either strain of *P. burhodogranariea* never showed *DptA* expression above what is seen for the sterile wound treatment at any time point (Fig. 2B).

Providencia induction of *Drs* over sterile wound was generally much smaller, and none of the infection treatments differ significantly from the sterile wound until 32 hours post infection (Suppl. Fig. 4A). The pattern of *Def* expression was more complex, with strong induction in response to *P. rettgeri* and *P. alcalifaciens* infections at 18 and 24 hours post infection (Suppl. Fig. 4B). The induction of *Def* in response to *P. sneebia* is much delayed relative to *P. alcalifaciens* and *P. rettgeri* infection, with strong induction not appearing until 32 hours post infection.

In summary, we observed that some of *Providencia* species that proliferate the most within the fly and cause the greatest host mortality also drive higher AMP expression. An

interesting departure from this trend is *P. sneebia*, which is highly virulent and reaches the highest abundance within the fly, but expression of *DptA* caused by *P. sneebia* infection is never significantly higher than that caused by a sterile wound (Fig. 2B).

3.4. Coinfections with *P. rettgeri* and *P. sneebia*

P. sneebia could avoid inducing a strong immune response by actively evading detection by the host or by actively suppressing the immune response. To distinguish between these two possibilities, we took advantage of the differences in mortality and immune induction resulting from *P. rettgeri* and *P. sneebia* infections. We coinfected flies with both bacteria simultaneously and then measured AMP expression, host mortality, and bacterial load. We hypothesized that if *P. sneebia* actively suppresses the immune response, we would see low levels of AMP expression even in the presence of *P. rettgeri*. Alternatively, if *P. sneebia* is not detected by the immune system, we would expect to see high levels of AMP expression induced by the presence of *P. rettgeri* in the coinfection.

We measured *DptA*, *Drs* and *Def* levels in groups of flies either coinfected or infected with an individual bacteria at 6, 24, and 32 hours post infection by QPCR (Fig. 3A; Suppl. Fig. 5). Across all 3 AMPs, infection with *P. sneebia* alone caused a lower immune response than infection with *P. rettgeri* alone at 32 hours post infection (Fisher's combined probability, $p = 0.00061$), consistent with the results presented in the previous section. Expression of the 3 AMPs at 32 hours post infection in coinfected flies was not significantly different from expression in flies singly infected with *P. rettgeri* (Fisher's combined probability, $p = 0.528$), but coinfected flies had significantly higher expression than flies infected with *P. sneebia* (Fisher's combined probability, $p = 0.014$). This result was further supported by visually examining the level of GFP expression in individual *DptA*-GFP flies, in which coinfected and *P. rettgeri* infected flies fluoresced more intensely than flies infected with *P. sneebia*. These data show that the lower expression of AMPs in flies infected with *P. sneebia* alone is not due to suppression of the immune response.

Consistent with previous mortality measurements (Fig. 1A), approximately 40% of the flies infected with *P. rettgeri* alone died from their infections, whereas about 95% of the flies infected with *P. sneebia* died within 72 hours (Fig. 3B). Coinfected flies exhibited 85% mortality. While all three treatments are significantly different than each other (in all cases $p < 0.01$), the overall mortality of coinfected flies is clearly more similar to that of flies infected with *P. sneebia* alone (Fig. 3B). When bacterial load was measured at 32 hours post infection, we observed that the abundance of each individual bacterium in the coinfected flies was not significantly different than their levels in flies that are singly infected (Fig. 3C; in all cases $p > 0.05$). Thus, it appears that the growth trajectories of the bacteria are completely independent of each other, and coinfected flies carry a bacterial load equivalent to the sum of each single infection. Considering all three coinfection phenotypes together, it is evident that *P. sneebia* is not able to suppress the host immune response, but is able to proliferate and cause host mortality even in the presence of an immune response triggered by *P. rettgeri*.

3.5. Biofilm formation

One way that *P. sneebia* could protect itself from recognition and the microbicidal AMPs is by forming a biofilm within the fly [34]. The fly would only be able to detect bacteria at the perimeter of the biofilm, and thus the magnitude of the immune response would not be proportional to the total number of bacteria present. Additionally the bacteria within the biofilm would be able to freely multiply without being affected by expressed AMPs. We tested all of our *Providencia* isolates for their ability to form biofilms *in vitro* in 96-well plates. *E. coli* was used as a control that can form a biofilm [35]. None of our *Providencia*

isolates formed a biofilm (Table 2). However, we cannot definitively rule out the possibility that *P. sneebia* might form a biofilm within the fly, since there could be host-specific molecules that act as signal to trigger *P. sneebia* biofilm formation *in vivo*.

3.6. Antibiotic protection assay

Another way that *P. sneebia* could evade detection and proliferate would be if it were able to invade cells and replicate within them. Strains of *P. alcalifaciens* that were isolated from human patients with diarrhea have been shown to invade human cells, demonstrating that some *Providencia* are able to do the first step in this process [16, 17, 19, 20]. We used an antibiotic protection assay to test whether *P. sneebia* is able to divide within *D. melanogaster* cells. We also tested whether *P. alcalifaciens* and *P. rettgeri* are able to divide within *D. melanogaster* cells since they proliferate within the fly during infection. *E. coli* was used as a negative control bacteria that would be passively phagocytosed by the cells but is unable to replicate within. *Listeria monocytogenes* was used as a positive control that is able to replicate within insect cells [36]. Bacteria were exposed to a phagocytic *D. melanogaster* cell line for two hours before antibiotics were added to the media to kill all extracellular bacteria. At 0, 6 and 24 hours post antibiotic killing of extracellular bacteria, both the media and the cells were plated separately and CFU were counted. The CFU found in the media were minimal by comparison to those within the cells. Since *E. coli* will only be passively phagocytosed by the cells, it was used as a standard for determining if any of our strains are actively invading the cells. Among all replicates, there were consistently fewer *P. sneebia* and *P. rettgeri* than *E. coli* inside host cells at the zero hour time point (Table 3), suggesting that these bacteria have some resistance to phagocytosis by these *D. melanogaster* cells. By contrast, *P. alcalifaciens* had higher numbers of CFU than *E. coli* at the initial time point suggesting that our strain of *P. alcalifaciens* is invasive. The positive control, *L. monocytogenes*, was able to replicate to high numbers within the cells. *P. rettgeri*, *P. sneebia*, *P. alcalifaciens*, and the negative control, *E. coli*, all had fewer intracellular CFU 24 hours after addition of antibiotic than at 0 hours, indicating that none of them are able to replicate within the insect cells. These data suggest that *P. sneebia* does not avoid recognition by the immune response by invading and proliferating in *D. melanogaster* cells.

4. Discussion

We have established that closely related bacteria in the genus *Providencia* vary in their pathology in a natural host, *Drosophila melanogaster*, as measured by the amount of mortality they cause, their ability to replicate within the host and the magnitude of the host immune response to their presence. Those bacteria which are able to grow most effectively in the fly often also trigger the most robust immune response and result in the most host death. However, one of these bacteria, *P. sneebia*, causes nearly complete mortality and quickly replicates to high numbers within the fly but does not induce a strong immune response. Through coinfections with the less virulent *P. rettgeri*, we concluded that *P. sneebia* is able to actively avoid detection by the immune system as well as protect itself from the immune response. We did not find evidence that *P. sneebia* forms a biofilm or replicates intracellularly *in vitro*. Although we were unable to determine the exact virulence mechanisms used by *P. sneebia* during infection of *D. melanogaster*, our data imply that *P. sneebia* implements more complicated or multiple strategies to subvert the immune system.

We note that the proportion of flies that die from each bacterial infection in the mortality assays is approximately equivalent to the proportion of flies that sustain high numbers of bacteria in the load experiments, suggesting that the individual flies in which *Providencia* is able to replicate are those that succumb to the infection. The data we have for flies infected with *P. burhodogranariea* strain B does not conform to this hypothesis, as that bacterium

causes a moderate amount of mortality despite not replicating within the fly as much as the similarly virulent *P. rettgeri*. This suggests that *P. burhodogranariea* strain B might do proportionally more damage to the fly, possibly by producing a harmful compound, at a lower density than the other bacteria. This also points at a distinction between the two *P. burhodogranariea* strains, as they both have similar levels of bacteria present during the first 32 hours of infection but strain D causes significantly less mortality. The two strains are defined as distinct based on differences in metabolic profiles and in sequence of some housekeeping genes [3]. Our data suggest there are likely to be further genetic differences between the strains, including in genes involved in the phenotypes examined here.

Although we are primarily interested in *Providencia* species that are natural pathogens of *D. melanogaster*, we also examined mortality due to infection by other species in the genus, which have been isolated as clinical infections of humans and other animals, or in one case, *P. vermicola*, as an associate of entomopathogenic nematodes [12, 13, 37]. The only bacteria we found to cause high mortality in infected *Drosophila* are those which were originally isolated from wild caught *D. melanogaster*. Two of these species were also previously described as clinical pathogens of humans, but in both of these species, *P. alcalifaciens* and *P. rettgeri*, the Dmel strain isolated from *D. melanogaster* caused greater mortality than the Type strain of the species. These results suggest that *Providencia* strains may become highly adapted to the host species they infect, and that the isolates recovered from *D. melanogaster* may be genetically suited to infect *Drosophila* and its close relatives. More detailed genomic and pathological examination of *Providencia* should reveal genes specifically involved in virulence to *Drosophila*.

Because we are specifically interested in the *D. melanogaster-Providencia* interaction after infection has occurred, we have relied on artificial infections to deliver the bacteria. Nevertheless, it is worth considering how the bacteria may establish infections in the wild. Our *Providencia* isolates do not cause mortality after being fed to flies in reasonable doses in the laboratory (data not shown), so it is not likely that they orally infect flies in the wild unless they are aided by coinfectors [e.g. 38, 39]. There is good reason to believe, however, that our method of infecting through a pinprick wound may mimic infections that wild *D. melanogaster* can receive [40]. Wild caught flies often have melanization independent of natural pigmentation patterns, indicating healed wounds, and frequently carry ectoparasitic mites that could be the cause of some of these wounds (unpublished observation). Mite wounds in honey bees have been shown to be secondarily colonized by environmental bacteria [41]. *P. burhodogranariea* strain B has been isolated from a mite pulled from a wild caught *D. melanogaster* (P. Juneja, personal communication), suggesting that mites may also directly vector bacterial infections, although fly-to-fly transmission of *Providencia* via mites has not been experimentally demonstrated.

We anticipate that *D. melanogaster-Providencia* system will be an excellent one for continued examination of many aspects of host and pathogen interactions. There is ample phenotypic diversity in the host-pathogen interaction, with clear variation among *Providencia* species in pathological phenotypes. Both the bacteria and the insect host can be easily and inexpensively manipulated in the lab, providing a valuable setting to conduct research that will not only give insight into interactions specific to this host-pathogen pairing, but also into generic virulence mechanisms and their genetic basis. *D. melanogaster* has been extensively studied as a generic host for pathogenic bacteria and a model for innate immune system function, and these *Providencia* isolates now provide an opportunity to study how flies fight those bacteria that infect them in their natural environment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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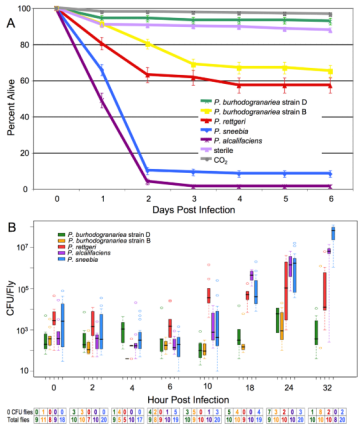

Figure 1.

Mortality of and bacterial proliferation in *D. melanogaster*. (A) Mortality of *D. melanogaster* from *Providencia* Infection. Wild type *D. melanogaster* were infected through pinprick infections with different strains of *Providencia.* All treatments result in highly significant differences in mortality (all pairwise contrasts $p < 0.0001$), except the difference between sterile needle and CO_2 controls, between infection with *P. burhodogranariea* strain D and either control, and between *P. rettgeri* and *P. burhodogranariea* strain B (in all cases $p > 0.0025$, the Bonferroni corrected cut off value). (B) *Providencia* Bacterial Load in *D. melanogaster*. Boxplot of the number of CFU present in *D. melanogaster* during the first 32 hours post infection. Note that the y-axis is a log scale. Whiskers approximate two times the standard deviation. The table under the graph has the number of flies that had no CFU at each time point for each treatment, as well as the total infected flies per treatment at each time point. Flies with no CFU present were not included in the boxplot. Sterilely wounded control flies never had any CFU at any time point.

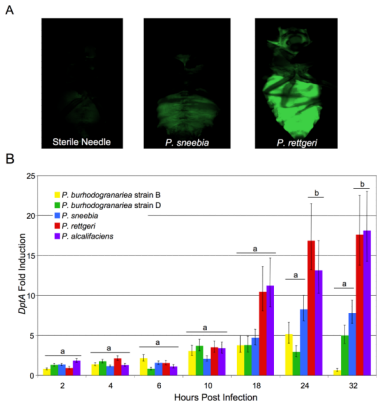

Figure 2.

DptA Expression in Flies Infected with *Providencia.* (A) *DptA*-GFP flies infected with (left to right) a sterile needle, *P. sneebia*, or *P. rettgeri* at 32 hours post infection. (B) Graph of *DptA* expression as measured by QPCR. The fold induction was calculated as the level of expression above that caused by a sterile wound alone. Error bars represent the standard error. At each time point, treatments labeled with “a” are not significantly different from the sterile wound alone while those with “b” are significantly different from the sterile wound (corrected for multiple tests by Tukey-Kramer method, cut off p=0.05).

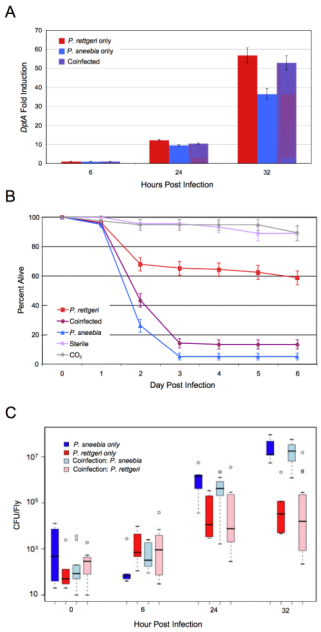

Figure 3.

D. melanogaster Coinfected with Both *P. sneebia* and *P. rettgeri*. (A) Graph of *DptA* expression from infection with *P. sneebia*, *P. rettgeri* or both measured by QPCR. The fold induction was calculated as the level of expression over that caused by a sterile wound alone. Error bars represent the standard error. The AMP genes *Def* and *Drs* showed similar patterns (Suppl. Fig. 4.) (B) Survival of *D. melanogaster* from infections with *P. sneebia*, *P. rettgeri* or both. (C) Bacterial load of *D. melanogaster* infected with *P. sneebia*, *P. rettgeri* or both. The two bacteria are plotted separately in pale colors for the coinfected flies. Whiskers approximate two times the standard deviation. Coinfected flies show full induction of the immune system, but succumb to their infections and permit bacterial growth that is not different than what is observed in single infections.

Table 1

Bacterial Strains Used. (*) indicates the strains that are the main focus of this work.

Species	Strain	DSM #	Isolated from	Citation
Providencia burhodogranariea	Type/B*	19968	wild *D. melanogaster* hemolymph	[2, 3]
	B97		wild *D. melanogaster* hemolymph	[2, 3]
	B18		wild *D. melanogaster* hemolymph	[2, 3]
	D*		wild *D. melanogaster* hemolymph	[2, 3]
Providencia rettgeri	Dmel*		wild *D. melanogaster* hemolymph	[2, 3]
	Type	4542	fowl cholera	[12]
Providencia alcalifaciens	Dmel*		wild *D. melanogaster* hemolymph	P. Juneja and S. M. Short, unpublished
	Type	30120	human infant dysentery	[12]
Providencia sneebia	Type*	19967	wild *D. melanogaster* hemolymph	[2, 3]
	A16		wild *D. melanogaster* hemolymph	[2]
	A36		wild *D. melanogaster* hemolymph	[2]
	A75		wild *D. melanogaster* hemolymph	[2, 3]
	A83		wild *D. melanogaster* hemolymph	[2]
	A91		wild *D. melanogaster* hemolymph	[2, 3]
	A101		wild *D. melanogaster* hemolymph	[2, 3]
	A102		wild *D. melanogaster* hemolymph	[2, 3]
	A104		wild *D. melanogaster* hemolymph	[2]
Providencia heimbachae	Type	3591	penguin feces	[12]
Providencia stuartii	Type	4539	human	[12]
Providencia vermicola	Type	17385	entomopathogenic nematode	[37]
Providencia rustigiannii	Type	4541	human feces	[13]

Table 2

Providencia isolated from *D. melanogaster* are not able to form biofilms. Measurements of absorbance of crystal violet at 540nm of replicate wells after biofilm formation *in vitro*. *E. coli*, which is capable of forming a biofilm, has much higher absorbance than any of the *Providencia* species.

	Relative biomass (A_{540nm})							
	without antibiotics				**with antibiotics**			
	6 hours		**24 hours**		**6 hours**		**24 hours**	
Bacteria	average	std dev	average	std dev	average	std dev	average	std dev
E. coli	0.0717	+/− 0.0277	0.2471	+/− 0.0929	0.0208	+/− 0.0054	0.0219	+/− 0.0021
P. sneebia	−0.0027	+/− 0.0047	0.0343	+/− 0.0036	−0.0023	+/− 0.0084	0.0125	+/− 0.0010
P. alcalifaciens	0.0039	+/− 0.0108	0.0261	+/− 0.0161	0.0007	+/− 0.0031	0.0058	+/− 0.0006
P. rettgeri	0.0056	+/− 0.0098	0.0277	+/− 0.0020	0.0117	+/− 0.0112	0.0104	+/− 0.0041
P. burhodogranariea strain B	0.0043	+/− 0.0002	0.0258	+/− 0.0063	0.0030	+/− 0.0074	0.0065	+/− 0.0001
P. burhodogranariea strain D	0.0048	+/− 0.0083	0.0127	+/− 0.0028	0.0060	+/− 0.0006	0.0121	+/− 0.0040

Table 3

Providencia isolated from *D. melanogaster* are not intracellular pathogens. Number of CFU within *D. melanogaster* S2 cells at 0, 6, and 24 hours post antibiotic killing of extracellular bacteria. Two replicate wells were measured each day and the experiment was carried out on multiple days with similar results. *Listeria monocytogenes*, which is capable of intracellular invasion and proliferation, grows to high density within host cells, whereas *Providencia* and *E. coli*, which is not capable of intracellular invasion, are progressively eliminated.

	well	0 hour CFU	6 hour CFU	24 hour CFU
P. sneebia	1	543	468	28
	2	466	460	22
P. rettgeri	1	285	110	49
	2	138	120	88
P. alcalifaciens	1	2541	2988	1620
	2	2265	2061	1170
E. coli	1	1734	942	154
	2	1500	465	105
L. monocytogenes	1	7570	8617	109680
	2	7145	14749	141200
PBS		0	0	0