Investigating a Small Colony Variant of *Providencia rettgeri* and Its Relation to Persistent Infection in *Drosophila melanogaster*

Honors Thesis
Presented to the College of Agriculture and Life Sciences, Microbiology
of Cornell University
in Partial Fulfillment of the Requirements for the
Research Honors Program

by
Miguel Gomez

May 2019

Dr. Brian Lazzaro

Abstract

Chronically persistent bacterial infections are an enormous health problem and are prevalent in many medical settings. Often, the bacteria that cause these infections exhibit a small colony phenotype, grow slowly, produce biofilms and are resistant to antibiotics. In this study, I used *Providencia rettgeri,* an opportunistic human pathogen and a natural pathogen of *Drosophila melanogaster.* From previous research in the Lazzaro Lab, it was found that *Providencia rettgeri* also has a small colony variant (S strain) phenotype. Therefore, I hypothesized that our isolated S strain is a canonical small colony variant with an ability to persist in the host, *Drosophila melanogaster.* After conducting various experiments to determine the growth dynamics, biofilm capabilities and antibiotic resistance of the *Providencia rettgeri* S strain, I concluded that the S strain (small colony phenotype) of *Providencia rettgeri* is most likely not a suitable model strain for studying small colony variants and their implication in persistent infections. This is because although the S strain did exhibit slower growth and diminished host lethality compared to the wildtype strain (L strain), its ability to produce biofilms and its antibiotic resistance were either equal to or worse than the L strain. Although this strain may not be a canonical small colony variant, it's slow growth and ability to persist in the host represents an interesting physiology worthy of continued study. A rescue experiment is currently in the works to determine if an identified mutation in the penicillin binding protein gene in the S strain is responsible for the small colony/low host lethality phenotype. The wildtype version of this gene, which is involved in cell wall synthesis and where the gene product is a known target for antibiotics, is hypothesized to restore the large colony size and

advanced growth demonstrated by the wildtype strain when expressed in the mutant (S) background.

Introduction

Chronically persistent bacterial infections are an enormous problem for public health and can be impervious to treatment. Persistent infections tend to be prevalent in medical settings, especially with medical implantations such as pacemakers or urinary catheters and chronic health conditions such as cystic fibrosis (Tümmler, Weißbrodt, Rohde, Häußler, & Steinmetz, 1999). Nevertheless, the study of chronic infections *in vivo* is currently difficult due to the fact that there is a lack of inexpensive and easily monitored animal infection systems. The bacterium that will be used in this study, *Providencia rettgeri,* is an opportunistic human pathogen that was also discovered to be a natural pathogen of *Drosophila melanogaster,* the model host for this study (Galac and Lazzaro 2011). *Drosophila* flies present a desirable model for infection studies due to their genetic tractability, ease of handling, and homology to mammalian innate immunity (Lemaitre and Hoffmann 2007). Previous research in the Lazzaro lab has shown that *P. rettgeri* is able to exhibit a bimodal infection in *Drosophila melanogaster* (where a percentage of flies are killed while the remaining flies survive with a chronic infection), offering a promising host-pathogen dynamic for studying persistent infections (Duneau 2017). A small colony variant (S strain) of *Providencia rettgeri* was identified by a collaborating lab (Justin Buchanan and Kristi Montooth, University of Nebraska-Lincoln) after isolation from an *in vivo* infection in *Drosophila melanogaster.*

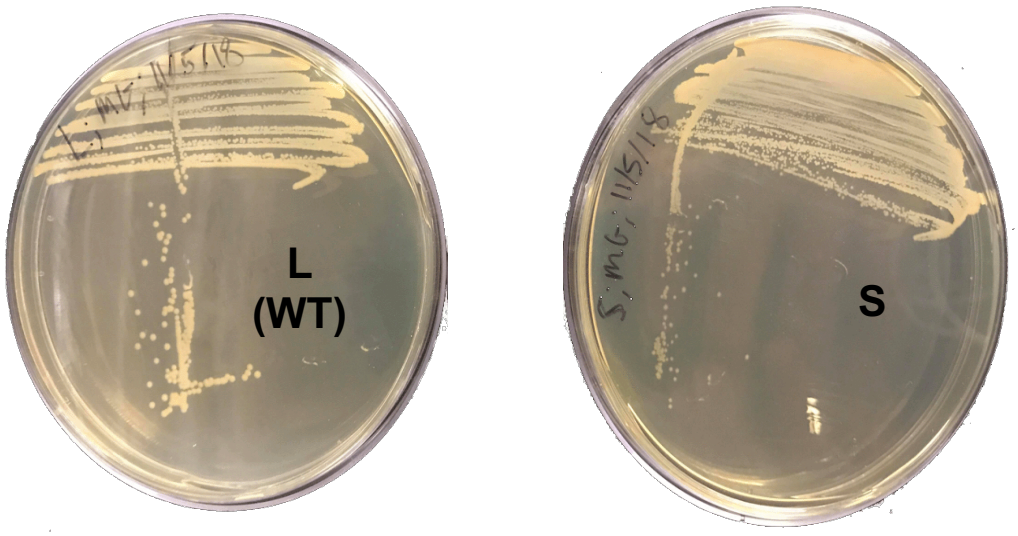


Figure 1- Colony morphology for both the wildtype (L strain) of *Providencia rettgeri* and the small colony variant (S strain) of *Providencia rettgeri.*

The mutant arose in the gut of a larval fly that had been fed with *Providencia rettgeri.* Similar to the wildtype (L, large colony) strain, the small colony variant is able to persist in the fly, although it does so with a lower bacterial burden (10^3 CFU/fly vs 10^5 CFU/fly for L strain) and kills fewer host flies upon reinfection (10-20% lethality vs. 50-60% lethality elicited L strain).

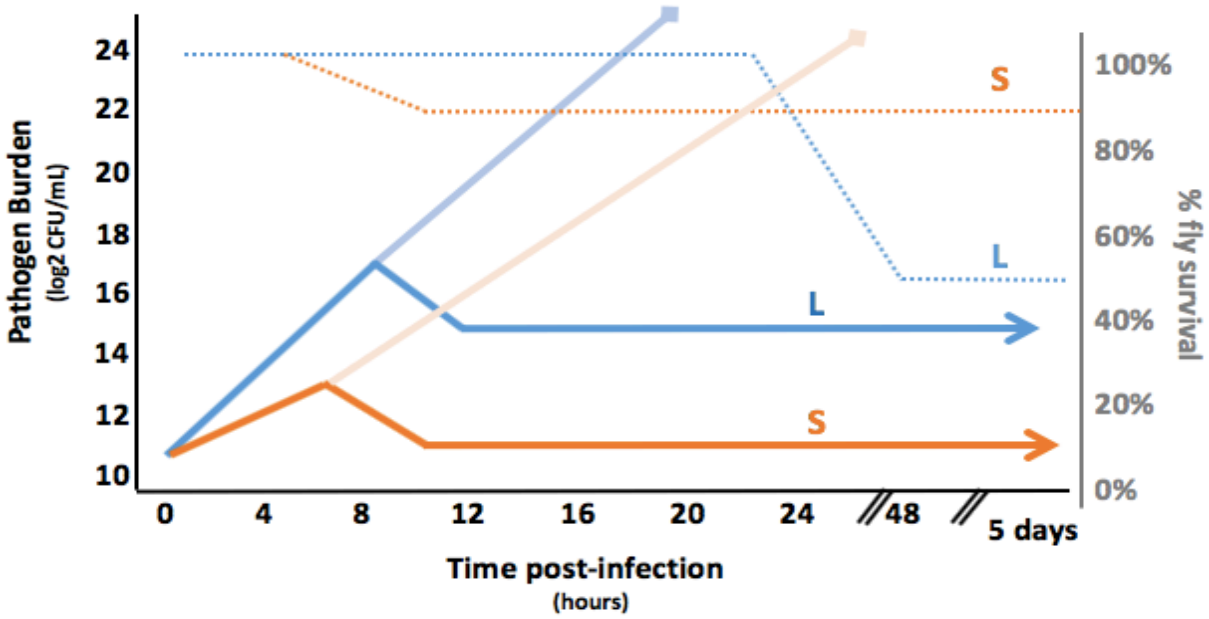


Figure 2- Bimodal infection kinetics of L and S strains measuring bacterial burden (left y-axis, black) and fly survival (right y-axis, gray) during infection of *D. melanogaster*. Solid lines represent pathogen burden (left y-axis) and dotted

lines represent fly survival (right y-axis). **Blue, L strain:** Thick blue line represents L strain chronic infection burden, lighter blue line represents L strain lethal infection burden and blue dotted line represents survival of flies when infected with L strain. **Orange, S strain:** Thick orange line represents S strain chronic infection burden, lighter orange line represents S strain lethal infection burden and orange dotted line represents survival of flies when infected with S strain.

Since small colony variants are often implicated in persistent infections, I was interested to determine if this S strain has any effect on persistent infections in *Drosophila melanogaster*. Canonical small colony variants have many common unique features including slow growth, ability to form biofilms and increased antibiotic resistance, which are all factors that could be implicated in the potential for the S strain to maintain a persistent infection (Santos and Hirshfield 2016). Given the preliminary data supporting its retention of persistence in the host, I hypothesized that our isolated S strain is a canonical small colony variant with an ability to persist in the host, *Drosophila melanogaster.*

Methods:

***In vitro* growth curves of S and L strains**

In vitro growth curves were performed in 96-well plates by measuring optical density (OD with absorbance wavelength of 600) over 24 hours using a SpectraMax 384 Plus (Molecular Devices) plate-reader. To initiate the growth curve, I inoculated 2 µL of an overnight culture of S or L into 198 µL of LB in wells of a clear flat-bottom 96-well plate (Globe Scientifics), resulting in a starting concentration of ~$2x10^7$ cells/well. A subset of wells was used as a control with only LB. I incubated the plate at 37°C with intermittent shaking for 24 hours. I acquired OD measurements every 15 minutes and plotted it as OD vs. time.

I also performed additional *in vitro* growth curves by culturing S and L strains in Erlenmeyer flasks containing LB. This approach allowed for samples to be extracted for both OD readings and viable counts (CFU/mL). I started flask cultures by transferring 1 mL of OD 1.7 S or L to a 500 mL flask containing 100 mL LB, resulting in a starting concentration of ~ $1x10^7$ cell/mL. Cultures were incubated at 37°C, 200 rpm for 8 hours. At each hour interval, I removed 400 µL from each flask used to measure OD while I removed another 100 µL and plated onto LB agar for viable counts using a spiral plater (Don Whitely Scientific).

***In vivo* growth curves of S and L strains**

I performed *In vivo* growth curves of the S and L strains by independently infecting flies with each strain and plating bacterial cells of infected flies over the course of infection. I counted the resulting colonies to determine bacterial load (colony forming units (CFU)/fly). The experiments were performed in duplicate in both wildtype Canton S *D. melanogaster*, as well as in Relish mutant flies, which have a severely diminished immune response (Ertürk-Hasdemir 2009). I infected Relish mutant flies and wildtype Canton S *D. melanogaster* flies with PBS (as a control), S strain or L strain (OD1 in PBS) using a pinprick infection (Khalil 2015) resulting in an initial administration of ~10^3 cells per fly. Fly survival was recorded over 72 hours by visual inspection. I measured bacterial load (CFU/fly) at 0, 4, 8, 24, and 48 hours by depositing single flies (10 per time point) into microfuge tubes containing 500 µL PBS and a 2.3 mm chrome steel bead (Biospec Products), homogenizing for 2 minutes (Talboys high throughput homogenizer), and plating onto LB agar with spiral plater (Don Whitely Scientific). Bacterial growth was plotted

as CFU/fly vs. time, which was calculated by taking the estimated CFU/mL value and multiplying it by 0.5 to adjust for the PBS volume that contained a single homogenized fly.

To determine whether a higher concentration of S would facilitate a more L-like infection (more lethality and higher bacterial burden in chronic phase), I performed *in vivo* infections with the S strain at increasing ODs (1, 2, 5 and 10) and monitored fly survival over 1 week. Fly survival from S strain infections were compared to infections with the L strain at OD1 (control) and flies infected with sterile PBS (control). I examined bacterial load immediately upon infection (T_0) to verify that infecting with increasing concentrations of S resulted in increasing bacterial load in the fly. I performed an additional experiment assessing bacterial load during the chronic phase (48 and 72 hours post infection) to determine if the higher doses of S increased fly lethality and bacterial load. For statistical analysis, I performed a Welch's two-sample t-test (unequal variance) for the *in vivo* infections in both Canton S *D. melanogaster* flies and relish mutant *D. melanogaster* flies. I also performed t-tests between the S and L strains to determine statistical significance between S and L bacterial load at 0, 4, 8, 24 and 48 hour marks post-infection (8-hour mark was excluded from relish mutant experiment). These were adjusted for a multiple-test correction. For the concentrated S infection experiment, I performed t-tests to determine statistical significance in bacterial load differences among different concentrations of S.

***In vitro* biofilm assay**

To determine the relative capability of the S and L strain strains to form biofilms, I used a staining method to quantify the biofilm size of each strain. I performed the assay according to

O'Toole 2011 (O'Toole 2011). Briefly, 1:100 dilutions of overnight S and L cultures were used to seed a 96-well plate that was then incubated at 37°C for ~24 hours. I washed wells twice by submerging that plate in water and stained attached cells by adding 125 µL of 0.1% crystal violet and incubating for 15 minutes at room temperature. I washed the plate again three times and dried before solubilizing the stained cells with 125 µL ethanol for 15 minutes at room temperature. The solubilized crystal violet was then transferred to a new 96-well plate and quantified by measuring absorbance at 550 nm on the SpectraMax plate reader. LB only wells were used as a blank. For statistical analysis, I performed a Welch's two-sample t-test between the S and L strain to determine statistical significance between S biofilm formation and L biofilm formation.

Minimum inhibitory concentration assay

I also performed Minimum Inhibitory Concentration (MIC) experiments on both S and L strains to determine the relative resistance to different doses of antibiotics between the two strains. Ampicillin (500, 400, 200, 50 µg/ml), tetracycline (200, 100 and 10 µg/ml) and kanamycin (100, 50 and 10 µg/ml) were used in the MIC experiments. I started MIC cultures by inoculating 40 µL of overnight cultures of S and L into 4 mL of LB containing the above antibiotics, resulting in a starting cell density of ~10^7 cell/mL. Cultures were incubated at 37°C, 200 rpm overnight. I qualitatively assessed growth through visual inspection of turbidity after ~18 hours. The MIC experiment was set up in duplicate where each antibiotic concentration was represented twice per strain.

Genome analysis for mutations in S strain

The genomes of the L and S strains were sequenced (Illumina) and alignments were generated prior to the start of this project (Gabe Fox and Ashley Frank, Lazzaro Lab). Candidate S strain mutation(s) potentially responsible for the small colony/low burden chronic infection phenotype were identified by comparing L and S strain genome sequences alignments in Geneious 10.2.3. Briefly, Illumina sequence reads were checked for quality with FastQC (Babraham Bioinformatics group) and adapters were trimmed using BBDuk. Reads from both S and L were aligned to the *P. rettgeri* reference genome in Geneious where single nucleotide polymorphisms (SNPs) were called using the software's SNP algorithm. This genome sequence comparison revealed two potential differences leading to the S strain's small colony/low burden infection phenotype. These are a C→T transition mutation in a penicillin binding protein (PBP) gene and a truncation in a lipoprotein gene. Of these, the PBP gene serves as my primary candidate for influencing the observed S phenotype due to its potential involvement in cell wall synthesis and antibiotic resistance, which is known to be a relevant phenotype for persisters.

Rescue of S strain with wildtype PBP

To confirm the candidate PBP mutation truly exists in the S strain and was not an artifact of Illumina sequencing, I amplified a 325 bp region of the PBP gene harboring the putative mutation in the S strain by PCR and verified the polymorphism with Sanger sequencing (Cornell Biotechnology Resource Center). I also performed parallel amplification and sequencing on the L strain to assure the mutation was unique to the S strain. The region was amplified with primers PBP_SNP_F: GTCACTTGGCCGCTTAAATC and PBP_SNP_R: GGCCTATTACCGTCGAATCT.

The 25 µl PCR reaction mixtures for this amplification consisted of 1x GoTaq buffer (Promega), 200 µM deoxynucleotide triphosphates (dNTPs) (Promega), 0.2 µM F and R primers (Integrated DNA Technologies), 0.025 U/µl GoTaq Polymerase (Promega), 100 ng genomic DNA. I amplified DNA with the following thermalcycling conditions: 95°C for 2 min, followed by 30 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 30 sec, with a final extension at 72°C for 5 minutes. I verified the PCR product by running samples on a 1% agarose gel. The product was cleaned with the Qiaquick PCR Purification kit (Qiagen) and Sanger sequenced at Cornell's Biotechnology Resource Center.

Once the mutation was confirmed by sequencing, I conducted PCR again to amplify the entire PBP gene (2,321 amplicon) from the L strain in order to get the desired wildtype product for a rescue study that involves expressing the *Providencia rettgeri* wildtype gene on a plasmid in the S strain mutant background (S-rescue). Due to the large size of the desired product, the PCR had to be optimized to increase yield and specificity. This was ultimately achieved through use of a high-fidelity polymerase, high GC polymerase buffer, elevation of annealing temperature (identified through gradient PCR), and use of DMSO. I amplified the entire gene with primers PBP_Full_F: AGACTTCAAAAGCAACCATATCT and PBP_Full_R: CACCTTCTAAAGCCCTTATATTGA. 50 µl PCR mixtures consisted of the following: 1x Phusion GC buffer (NEB), 200 µM dNTPs, 0.5 µM F and R primers, 3% DMSO, 0.02 U/µl Phusion Polymerase (NEB), 100 ng genomic DNA. DNA was amplified with the following thermalcycling conditions: 98°C for 30 sec, followed by 30 cycles of 98°C for 10 sec, 59.3°C for 30 sec, 72°C for 2 min, with a final extension at 72°C for 5 minutes.

Future experiments are planned to add adenines to the PBP gene amplicon for TA cloning into pCR2.1-TOPO (Invitrogen) and subcloning into the expression vector pBAD33mob (gift from Tobias Doerr, Cornell) through shared SalI and HindIII restriction sites. The expression construct will then be transferred to the S strain via conjugation and used to express the WT PBP gene in the S strain background to determine if this gene can restore the wildtype phenotype (large colony, enhanced growth rate, increased host lethality and burden during *in vivo* infection).

Results

***In vitro* growth characterization of S and L**

I used both 96-well plate and flask-based cultures to compare the relative growth kinetics of the S and L strain during *in vitro* incubation. Both methods yielded the consistent result that the S strain grows more slowly and at a lower endpoint density than the wildtype strain (Figures 3 and 4).

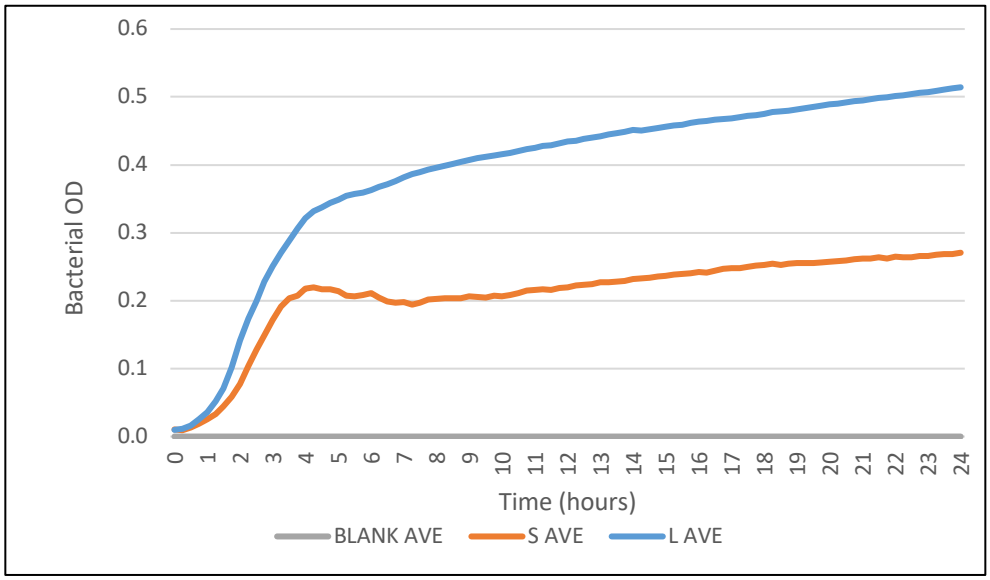


Figure 3 – *In vitro* growth curve performed in 96-well plate comparing S strain (small colony variant), L strain (wildtype) and a negative control of a blank (LB only). Bacterial OD was measured at a wavelength of 600 nm. Growth was measured for 24 hours. Lines represent average of 8 wells per strain.

A) B)

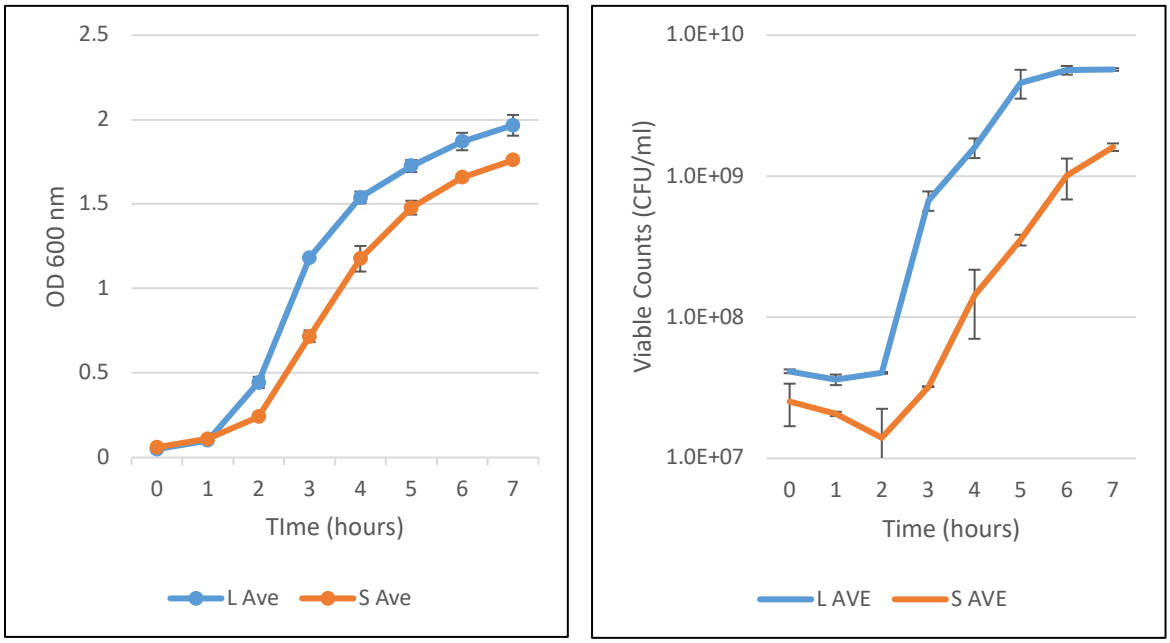


Figure 4- Flask-based growth curve measurements using (**A**) optical density and (**B**) viable counts measured in colony forming units (CFU)/mL. Error bars account for variation between 3 replicates.

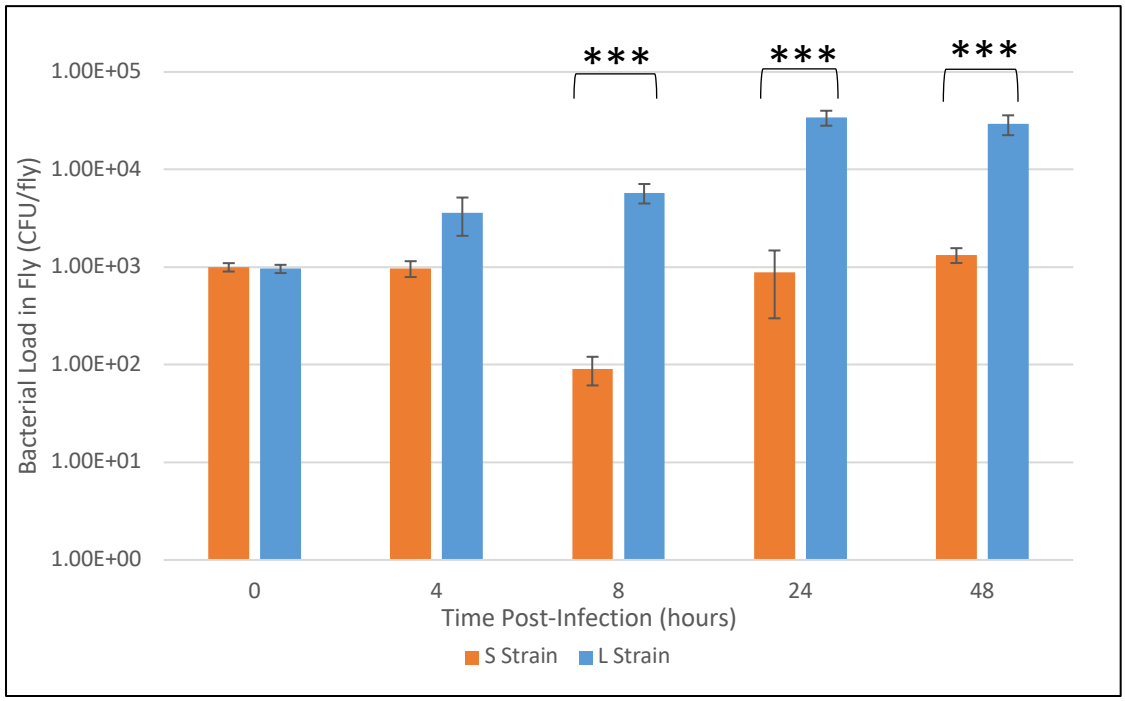
Although the flask-based curve was not extended long enough to determine cell density during stationary phase, both the plate and flask curves indicate that L reaches a higher OD than S (L max plate OD = 0.51, S max plate OD = 0.27; L max flask OD = 2, S max flask OD = 1.75) and grows at a faster rate (L plate growth rate = 0.12 OD/hr, S plate growth rate = 0.08 OD/hr; L flask growth rate = 0.74 OD/hr, S flask growth rate = 0.47 OD/hr). The viable counts measured during the flask-based growth curve provide a more direct estimate of viable cell number (as OD is dependent on cell size, shape, and health), and support the results from both OD growth curves, demonstrating slower and lower growth by the S strain (L growth rate = 7.8×10^8 CFU/mL/hr, S growth rate = 3.2×10^8 CFU/mL/hr) (Figure 2B). Additionally, I examined cells from

cultures of S and L under a compound microscope to assure that the difference in optical density was not merely due to a smaller physical cell size of S cells. Upon visualization at 1000x total magnification, no difference in cell size was observed (data not shown). Based on the data presented in Figures 1 & 2, my hypothesis that the S strain has slower growth compared to the L strain is supported.

***In vivo* infections and survivorship curves for S vs L**

As an initial measurement of relative virulence during infection, I infected flies with either S or L strain via septic pinprick and monitored for fly survival (Figure 5A) and bacterial load (Figure 5B) over time. While both strains exhibit a bimodal infection where a percentage of flies are killed and the remaining survive with a chronic infection, the S strain appears to demonstrate less direct virulence (lower fly mortality and lower bacterial burden during chronic infection). The differences between the S and L infection start to be apparent around the 8 hour mark and solidify by the end of experiment when only 40% of the flies infected with L survive and harbor high bacterial loads (4.8×10^4 cells/fly), whereas 85% of the flies infected with S survive with a lower bacterial load (1.7×10^3 cells/fly).

A)



B)

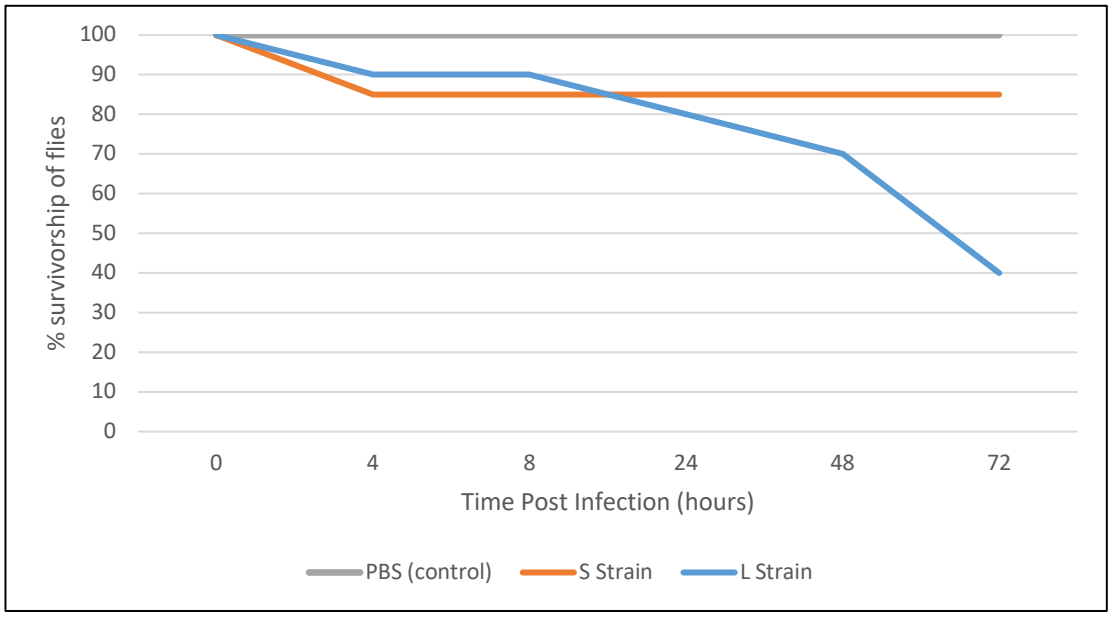


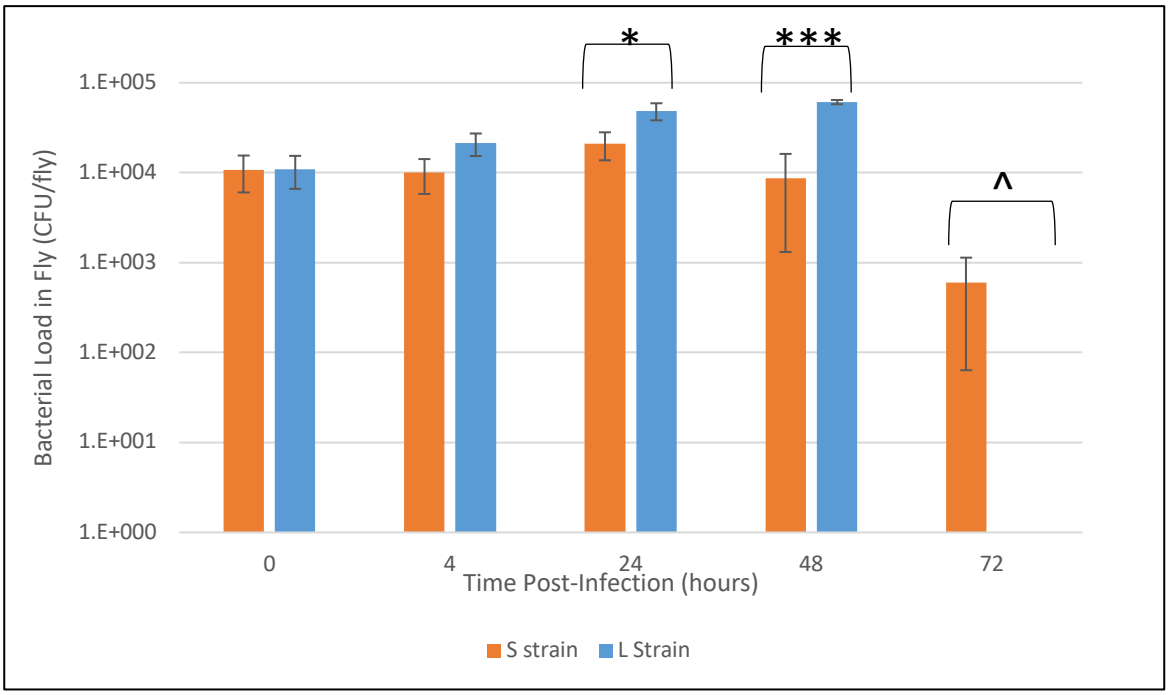
Figure 5– A) *In vivo* infections comparing bacterial load in Canton S. *Drosophila melanogaster* after infection with S or L strain of *Providencia rettgeri*. Bacterial load was measured and compared at 0, 4, 8, 24, and 48 hours post-infection. Error bars indicate variance between 3 replicates. ***= statistical significance at a p-value <0.005. B)

Survivorship curve of Canton S. *Drosophila melanogaster* after infection with S strain, L strain or PBS (control). Assay started with 30 flies per treatment.

While bacterial load stabilizes for both strains by 48 hours post-infection, the S strain is carried at a significantly lower load than the L strain (Figure 5A). Bacterial burden is statistically significantly different between infections with S and L strains at 8 hours, 24 hours and 48 hours post-infection. In addition, there is a drop in the bacterial load at the 8 hour post-infection mark for S strain (which occurred in all three replicates), which is not evident in the L strain.

It is known that *P. rettgeri* infections activate the fly immune system via expression of antimicrobial peptides through the Imd pathway, even throughout chronic infection, and that the absence of a functional Imd pathway results in 100% host mortality during WT *P. rettgeri* infection (Myllymäki 2014). I therefore wanted to assess if disabling the fly's Imd pathway would similarly allow for total (or increased) mortality during an S infection. To help determine whether the chronic infection of S is dependent on the host immune system, I repeated the *in vivo* infections using the Relish mutant of *Drosophila melanogaster.* Relish is an NF-kB transcription factor that controls expression of antimicrobial peptide genes of the Imd immune pathway. Flies that are mutant for the relish gene have diminished immune function (Ertürk-Hasdemir 2009).

A)



B)

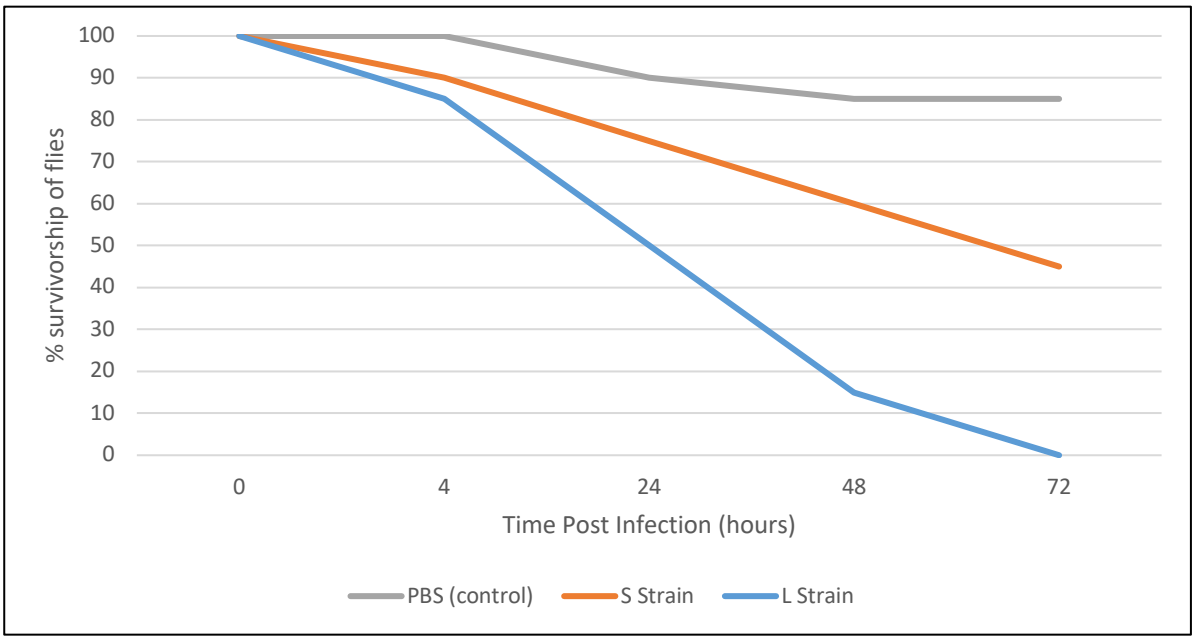


Figure 6– A) *In vivo* infections comparing bacterial load in Relish *Drosophila melanogaster* after infection with S or L strain of *Providencia rettgeri.* Error bars indicate variance between 10-30 replicates. *= statistical significance at a p-value <0.05, ***=statistical significance at a p-value <0.005, ^= no statistical comparison could be made since Relish fruit flies infected with L strain did not survive past 72 hour timepoint. B) Survivorship curve monitoring survival of Relish *Drosophila melanogaster* after infection with S strain, L strain or PBS (control).

In the absence of a fully functional host immune system, both strains are able to proliferate strongly for the first 24 hours (Figure 6A). After that point, all L-infected Relish flies die off and ultimately all die by 72 hours (Figure 6B), demonstrating higher mortality of the Relish flies (100% lethality) than wildtype flies (60% lethality) when infected with the L strain (Compare Figure 5B with Figure 6B). Similarly, the S-infection kills 40% more Relish flies than wildtype flies by 72 hours (compare Figure 5B with Figure 6B), with a continued lethal trajectory; however, the surviving S-infected Relish flies at 48 and 72 show a drop and leveling of bacterial load consistent with a chronic infection. Extended infection times and bacterial load sampling would be required to determine if the infection would persist indefinitely or ultimately cause 100% mortality. PBS control infections also led to higher death in Relish mutants than in wildtype controls (compare Figure 5B and 6B) suggesting that Relish flies are susceptible to death from injury due to lowered immune function.

As previously explained, flies infected with *P. rettgeri* are either killed by the highly proliferating bacteria, or survive with a life-long chronic infection (see Figure 5) (Duneau 2017). I observed that infection with the S strain exhibits a similar bimodal infection outcome, however, the flies that survive do so with an even lower bacteria load than flies infected with the L strain (Figure 5A). The low bacterial burden observed in the chronic S infection lead me to hypothesize that administration of a higher infectious dose of S would result in a more L-like infection phenotype (increased host lethality) in wildtype Canton S. *Drosophila melanogaster*. Before pursuing this, I first verified that use of increasing stock concentrations of S (OD1, OD2, OD5, OD10) for infection would result in a concomitant increase in T_0 bacterial load. Results

indicated that this method was reliable in transferring increasingly large amounts of bacteria to the fly, however, the percent increase was lower than expected for the percent increase in the cell stocks (data not shown). Although the increase in initial bacterial load was not exactly proportional to the increase in starting stock concentration, the general trend of increasing load with increasing cell stock concentration provided sufficient confidence to proceed with subsequent concentration-based experiments. We next performed the full experiment to monitor relative fly survival upon increasing infectious doses of the S strain (Figure 7).

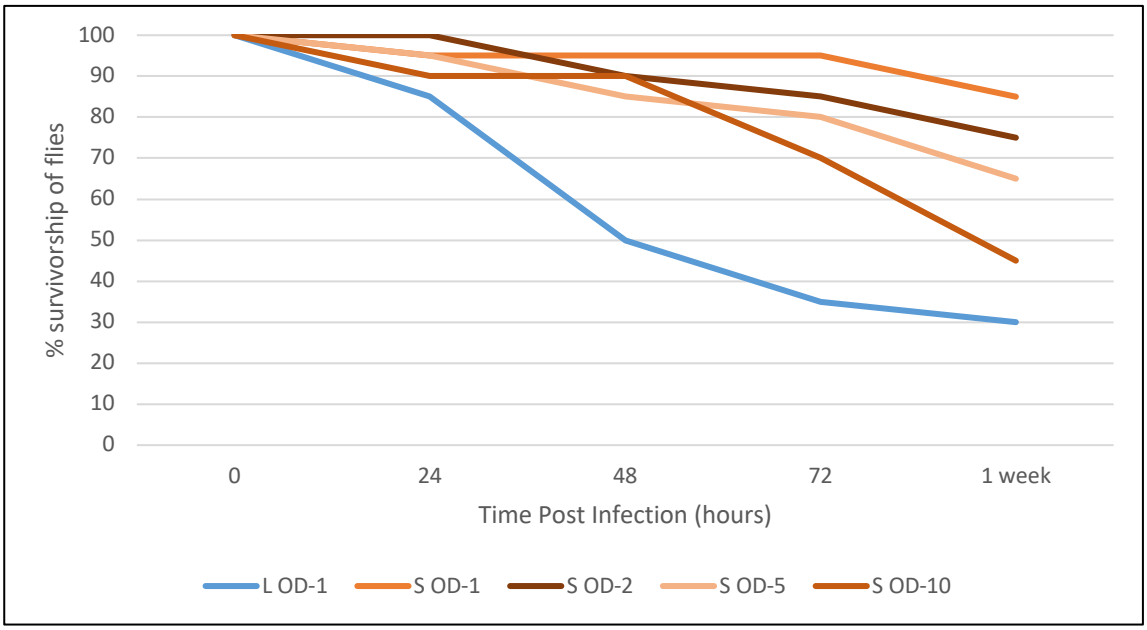


Figure 7. Survivorship curve monitoring survival of Canton S *Drosophila melanogaster* after infection with different concentrations of the S strain. S cell concentrations of OD 1,2,5, and 10 (corresponding to an initial does of ~3,000, 6,000, 15,000, and 30,0000 cells, respectively) were prepared from the same overnight culture by diluting cells to OD1, and then pelleting and resuspending in smaller volumes of PBS to generated the more concentrated stocks. OD1 of the L strain was used as a control.

When looking at the survivorship curve, as S strain dose increases, survivorship of fruit flies decreases. This is especially apparent 72 hours post-infection to 1 week post-infection (at 1 week post-infection, S OD 1 survivorship = 85%, S OD 2 survivorship = 75%, S OD 5 survivorship = 65% and S OD 10 survivorship = 45%). As S strain inoculation dose increases, survivorship is

closer to L OD 1 survivorship (30% after 1 week). Based on the results, we can see that a higher infectious dose of the S strain does in fact lead to a more L-like infection phenotype with increased host lethality. This supports my initial hypothesis that since the S strain is more susceptible to the fly immune system, a higher concentrations of cells would be required in order to overwhelm the immune system and cause greater rates of fly lethality. A follow up experiment should be performed where bacterial load per fly is analyzed for higher S concentrations up to 72 hours post-infection (0, 4, 8, 24, 48 and 72 hours) to determine if higher S concentration infections leads to bacterial loads similar to L infection.

***In vitro* Biofilm assay:**

Since biofilm formation is a common mechanism of persister cells, I evaluated the relative ability of S and L strains to form biofilms by an *in vitro* plate-based biofilm assay (O'Toole 2011). This assay stains the biofilm with crystal violet which is then solubilized in organic solvent for spectrophotometric measurement to quantify the intensity of the biofilm. I predicted that the S strain should be better at forming biofilms in comparison to the L strain if it is implicated in persistent infection within fruit flies.

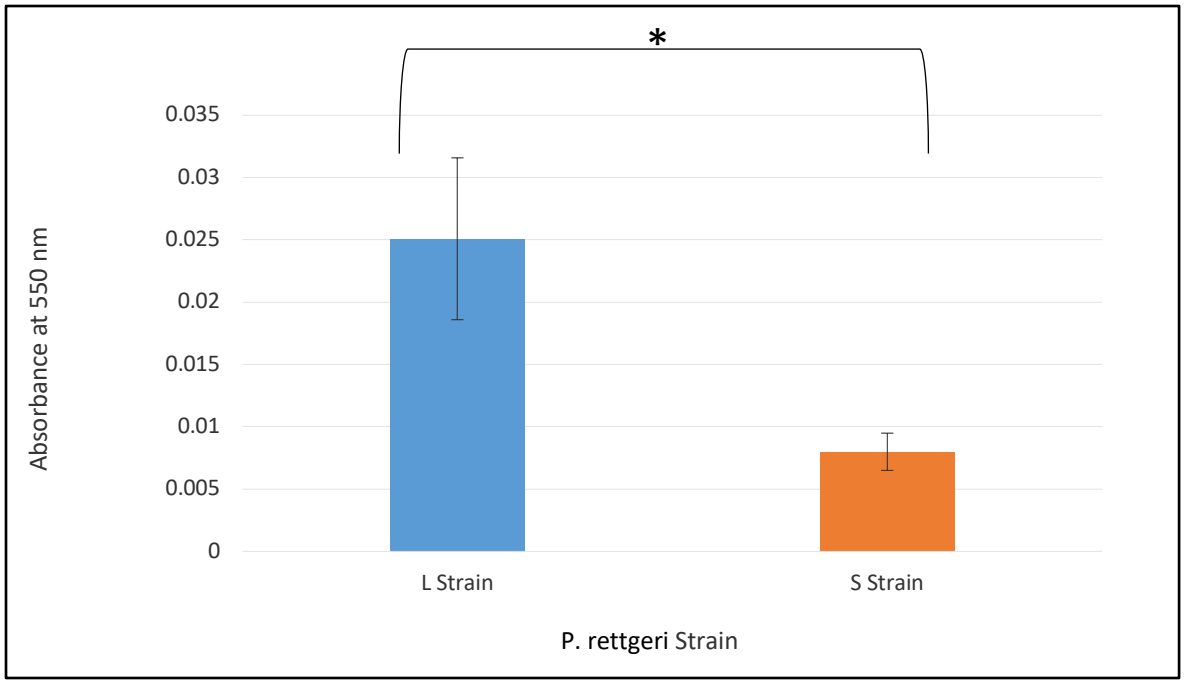


Figure 8- *In vitro* biofilm assay comparing S strain and L strain's ability to form a biofilm measuring crystal violet stained biofilm absorbance at 550 nm. Error bars indicate variance among 8 replicate wells. A control using only LB was performed and is accounted for in graph as a background where absorbance measurements from the control were subtracted from all L strain and S strain absorbance measurements. *= statistical significance at a p-value <0.05.

Data indicates that the stained biofilm absorbance was higher for the L strain (OD=0.025) than the S strain (OD=0.0075) and the difference was statistically significant (p = 0.05) (Figure 8). This fails to support my hypothesis that biofilm formation would be higher in the S strain culture. Since overall intensity of crystal violet staining were relatively low, it is worth repeating the experiment to see if higher maximum concentrations of L and S strain can be achieved during growth in the microtiter plate. Higher cell concentrations would allow for the bacteria to form biofilms more readily. This is because the larger the concentration, the more likely it is for bacteria, like *Providencia rettgeri,* to attach to a surface and communicate with other bacteria to do the same (Costerton, Stewart, & Greenberg, 1999). Repeating this experiment in a minimal medium like M9 may also help to facilitate stronger biofilm formation

for more robust quantification and thus higher crystal violet staining during the biofilm assay. This is because in order for the bacterial cells to survive in a minimal media such as M9, it would be advantageous for bacteria to form biofilms as the biofilms will trap essential nutrients and water in the environment to promote bacterial growth (Costerton et al., 1999).

Rescue of S strain with wildtype PBP:

By comparing L and S strain genome sequences, we identified S strain mutation(s) potentially responsible for its small colony and low burden infection phenotypes. One of the identified mutations was a C→T transition mutation in the 3' end of a PBP gene (Figure 9). This mutation was present in the Penicillin-binding protein's C terminal region. Due to the PBPs involvement in cell wall synthesis and interactions with antibiotics, it was identified as the primary candidate for the S strain phenotype, and thus was selected for complementation by the wildtype gene.

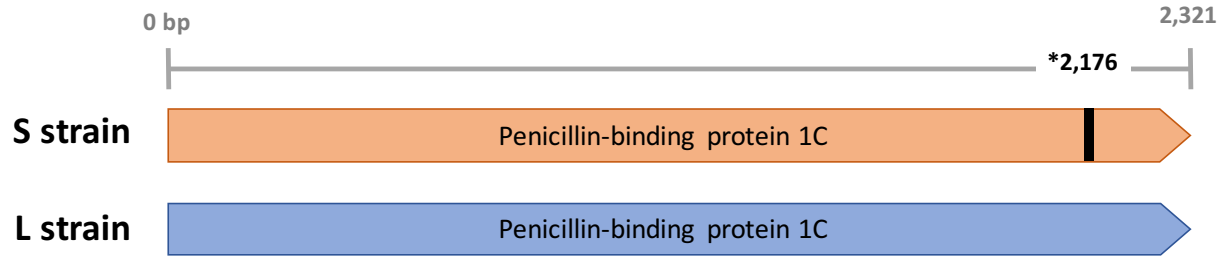


Figure 9- Genome comparison between L and S strain to identify the new mutation on the PBP gene in the S strain. The new mutation is circled.

To verify that the PBP SNP was present in the S strain (and not an artifact of Illumina sequencing), a portion of the gene harboring the putative mutation was PCR amplified and run on a gel (Figure 10A). I confirmed the SNP mutation using Sanger sequencing of the 325 bp amplicon with comparison to an equivalent product from the wildtype strain. Next, I performed

gradient PCR using Phusion polymerase in order to optimize conditions for isolation of the 2.5 kb wildtype PBP gene region from the L strain for the rescue study. Results from the gradient PCR suggest that the best annealing temperature for the PCR was 59.3 ^{0}C due to amount of pure product produced after running on an agarose gel (data not shown). I repeated PCR using this annealing temperature with the Phusion polymerase protocol to amplify sufficient product yield and specificity for use in the subsequent cloning project (Figure 10B). In future efforts, the isolated gene will be cloned into pCR2.1-TOPO, subcloned into the expression vector pBADmob and transferred to the S strain via conjugation for expression of the WT PBP in the mutant PBP background. Since the PBP is involved in cell wall synthesis, we predict that expression of the WT gene in the S background may restore colony size and infection phenotype (increased lethality).

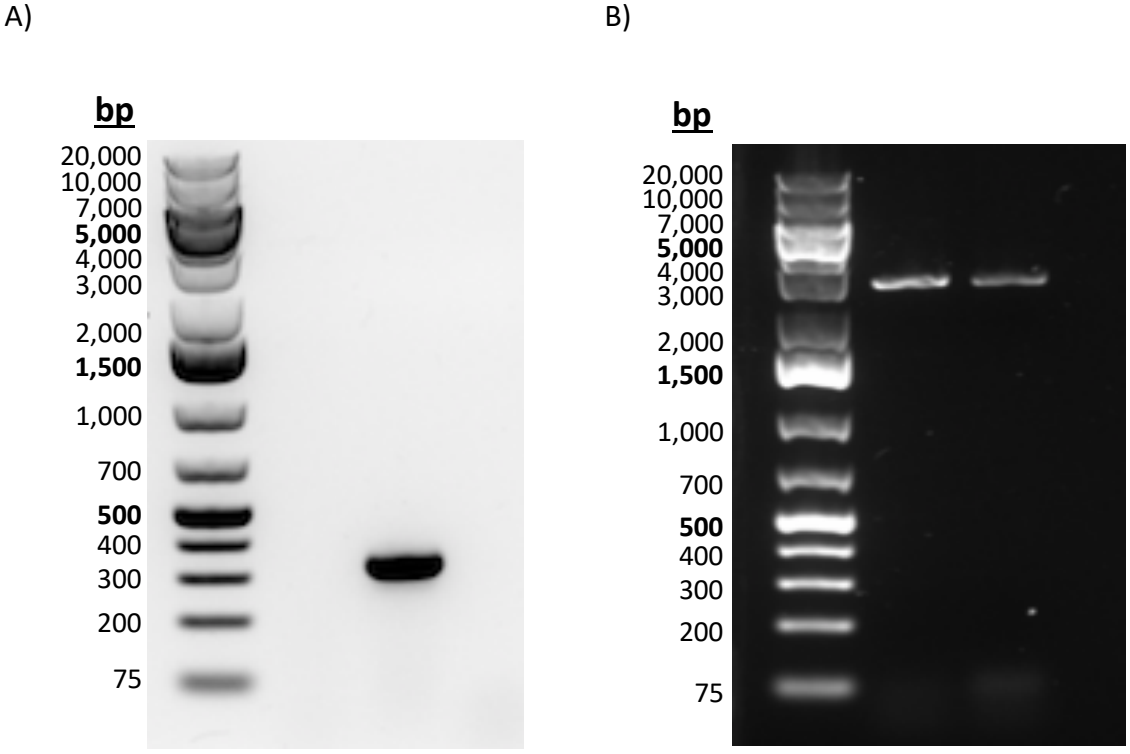


Figure 10- a) Agarose gel of the 325 bp region around the SNP of PBP gene from the S strain l. Wells, from left to right, are ladder, master mix with 1 ul of water (negative control) and PBP SNP S strain amplicon. b) Agarose gel of

the ~2.5-3 kb region encompassing the wildtype PBP gene. Wells, from left to right, are ladder, PBP gene product, replicate 1 and PBP gene product replicate 2.

Minimum inhibitory concentration assay:

Antibiotic resistance is a characteristic typically found in bacteria capable of persistent infection (Proctor, von Eiff, 2006). The S strain of *P. rettgeri* has a mutation in the penicillin-binding protein (PBP) gene. PBPs are involved in cell wall formation and thus critical to the maintenance of cell integrity. This integrity can be disrupted when PBPs are bound by ß -lactam antibiotics (like ampicillin and penicillin), ultimately leading to the cell death (Williamson 1987). Mutations of the PBP may help reduce its affinity for antibiotics, and therefore I hypothesized that the S strain harboring the PBP mutation would have increased antibiotic resistance to ampicillin compared to the L strain. I performed an MIC assay using ampicillin, kanamycin and tetracycline to determine antibiotic susceptibility differences between the L and S strain. Kanamycin and Tetracycline were used as controls as they both have different modes of action compared to ampicillin (kanamycin is an aminoglycoside antibiotic that targets the 30S ribosome while tetracycline is a protein synthesis inhibitor).

Table 1 – Minimum Inhibitory Concentration (MIC) assay performed comparing growth between S strain and L strain when provided different concentrations of antibiotics. **+** = growth **-** = no growth.

Antibiotic & Concentration (µg/ml)	S Strain	L strain
Ampicillin 500	-	-
Ampicillin 400	+	+
Ampicillin 200	+	+
Ampicillin 50	+	+
Kanamycin 100	-	-
Kanamycin 50	-	-
Kanamycin 10	+	+
Tetracycline 200	-	-
Tetracycline 100	+	+
Tetracycline 10	+	+

Both the S strain and L strain had the same MIC for kanamycin (50 µg/mL), tetracycline (200 µg/mL) and ampicillin (500 µg/mL) (Table 1). This opposes the hypothesis that the S strain would be more resistant to ampicillin (a ß-lactam) due to the mutation in its PBP. It should be noted that the ampicillin results for S and L strain were performed 4 times, as inconsistent results were obtained for the 500 µg/mL concentration, with S demonstrating resistance sometimes and susceptibility other times (resistance only occurred once (+) while susceptibility occurred twice (-).

Discussion

Small colony variants are implicated in many medically important infectious diseases that are capable of persistent infection. For example, patients suffering from cystic fibrosis tend

to develop persistent infections with pathogenic strains of *Pseudomonas aeruginosa* (Tümmler 1999). It has been noted that these bacteria that cause persistent infection do so due to their enhanced ability to produce biofilms, resist the effects of many antibiotics and grow slowly within their host (Santos and Hirshfield 2016). Thus, small colony variants need to be studied in order to improve available treatments for those suffering from persistent infections. In this study, I studied a small colony phenotype (S strain) for *Providencia rettgeri*, a pathogenic bacterial strain in *Drosophila melanogaster,* in order to determine whether it was a true small colony variant persister. This was done by first investigating whether the small colony mutation exhibited characteristics implicated in persistent infections (e.g slow growth, biofilm formation and increased antibiotic resistance) and then identifying potential genes that could account for persistent infection with the downstream intention of heterologously expressing the wildtype gene in the mutant background to determine if the wildtype phenotype is restored.

In this study, I performed *in vitro* and *in vivo* growth curves in order to identify whether the S strain exhibited slow growth, a characteristic linked to persistent infection and small colony variants. All the *in vitro* growth assays (both plate and flask assays) support that the S strain grows more slowly and to a lower density than the L strain (Figures 3-4). This is also mimicked in the fly, where chronic infection is maintained with lower loads than L strain (Figure 5). This slowed growth and maintenance of chronic infection is consistent with the persister phenotype; however additional experiments suggest the phenotype may be a simple growth defect and not related to canonical persistence. In previous work, it was found that slowed growth of persister staphylococcus is a necessary characteristic for small colony variants, observing that it is difficult for small colony variants to reach OD's above 2 to 3 after 24 hours

of incubation (von Eiff 1997). This is consistent with the results of our *in vitro* growth curves, where the S strain does not surpass an OD of 1.8. Other studies dealing with small colony variants also support the result that slow growth is a hallmark of persistent infection. For instance, it has been noted that small colony variants of staphylococcus grow at a slower rate in comparison to their wildtype counterparts and that this slow growth can be due to a variety of metabolic alterations including defects associated with the electron transport chain and the biosynthesis of metabolites (Proctor, von Eiff, 2006). The slowed *in vivo* proliferation of S relative to L is maintained in infection of Relish flies which have severely compromised immune systems (Figure 6). This means that regardless of immune function, the S strain will grow slower within the fly compared to the L strain. Since Relish flies do not have the capacity to clear themselves of infecting cells, the lower cell numbers of S truly represents a slower proliferation (and not enhanced clearance by the host). Based on the growth dynamics of the S strain of *Providencia rettgeri,* the S strain does exhibit slower growth, a characteristic implicated in small colony variants linked to persistent infection, however, it does not exhibit other characteristics of typical small colony variants, including enhanced biofilm formation and antibiotic resistance.

Due to its involvement in persistence, I compared biofilm formation between the L strain and S strain. Biofilm formation is a key virulence trait in many small colony variants and is heavily implicated in its ability to cause a persistent infection. This is because biofilms are relatively immobile, allow increased antibiotic resistance, and allow for exchange of beneficial genes between bacteria since they are closer together and adhere to nutrient rich surfaces within the host, which can allow the bacteria to persist within the host and resist innate host defenses (Costerton 1999). If the S strain were a true persister, it should produce more biofilm

than the wildtype strain. Contrary to this prediction, biofilm intensity was lower in the S strain (Figure 8) suggesting that it may actually be less capable of forming a biofilm. It would be ideal to do a biofilm assay comparing the S strain and L strain ability to make biofilms *in vivo* to get a more realistic interpretation of biofilm status during host infection. However, this would be challenging due to interference of the host tissue and the technical requirements needed to isolate biofilms from the host. To expand upon the biofilm analysis, more replicates should be done in order to measure the reliability of the initial results. In addition, the biofilm assay should be performed again using an increased incubation time in order to determine if it takes longer for the S strain to form a biofilm that exceeds that of the L strain.

Studies have shown that small colony variant persisters can tolerate higher concentrations of antibiotics compared to wildtype cells (Proctor, Kriegeskorte, 2014). Due to a mutation in the PBP gene of the S strain (Figure 9), I predicted that antibiotic resistance would only be greater for ß-lactam antibiotics (like ampicillin) in the S strain and that kanamycin and tetracycline susceptibility would be the same. However, in the MIC assay, I found that S strain and L strain had the same susceptibility to all three antibiotics and both strains were equally resistant to ampicillin (MIC of 500 µg/mL, Table 1). This also disproves the hypothesis that S strain would have increased antibiotic resistance to ampicillin if it is a true small colony variant persister. However, this result is not surprising since the S strain also does not have an enhanced ability to form biofilms (Figure 8), which can be responsible for the increased antibiotic resistance of canonical small colony variants. Although biofilm formation and antibiotic tolerance can be independent traits, the lack of both of these features in the S strain lends support against its capacity to act as small colony variant persister. However, the tests

that were performed (for both biofilm and MIC) were done outside of the fly and do not provide insight into the physiologies that would occur during an *in vivo* infection. It is therefore suggested to test ampicillin resistance during infection in the host. If the S strain demonstrates a higher tolerance of ampicillin during chronic infection than the L strain, then it may still be a candidate for a canonical persister cell.

In the initial stages of this study, candidate genes were identified that could potentially contribute to the S strain phenotype (small colony and slow growth). Upon genome comparisons between the S and L strains, two particular mutations were marked as high-priority candidates for investigation. These were mutations in the penicillin binding protein gene and a lipoprotein gene. Both genes are predicted to be important to pathogenicity since PBP is an important marker for antibiotic binding and lipoprotein is a membrane protein that serves as a recognizable antigen for the immune system of the host. Due to the PBPs involvement in antibiotic binding as well as its necessity for cell wall synthesis, it served as our primary candidate for further experimentation. While we have begun the molecular biology to clone the wildtype PBP gene into an expression vector, our ultimate goal is to express this wildtype gene on a pBAD vector (Guzman 1995) in the mutant background to determine if the wildtype phenotypes are restored (large colony, faster growth, higher fly lethality etc.) This will therefore demonstrate whether the mutation found in the PBP gene is an influential gene in the observed S phenotype. Expanding upon the rescue study described above, if that fails, a rescue study can be done using the lipoprotein gene instead of the PBP gene (mentioned in methods, p. 6) in order to see whether the lipoprotein gene is an influential gene in the observed S phenotype.

Conclusion

Based on the results accumulated in this study, I concluded that the S strain of *Providencia rettgeri* is not likely a canonical small colony variant with persistence in the *Drosophila melanogaster* host. This is because it does not have certain characteristics (biofilm formation, enhanced antibiotic tolerance) that would match its ability to persist within its host, *Drosophila melanogaster*. The S strain exhibited slow growth and decreased pathogenicity and was able to retain a persistent infection within its host; however, the S strain was not able to form biofilms as effectively as the wild type L strain and consequently had identical antibiotic resistance to the L strain. Since these are two features that are significant in most medically important small colony variants involved in persistent infection, I concluded that the S strain (small colony phenotype) of *Providencia rettgeri* is most likely not a suitable model strain for studying small colony variants and their implication in persistent infections. However, we believe this strain's interesting physiology (small colony and retained persistence in the host) is worthy of continued study as it may reveal novel insight into bacterial growth dynamics inside and outside of the host.

Acknowledgments

I would like to thank Dr. Brian Lazzaro for his mentorship and support throughout the project, allowing me to develop many research skills. I would also like to thank Ashley Marie Frank for her constant guidance throughout the project and for working with me and helping me with the project from start to end. I also would like to thank Dr. Joe Peters and Dr. James Shapleigh for being a part of my review committee and taking the time review and critique my thesis.

Citations

Costerton, J. W., Stewart, P. S., & Greenberg, E. P. (1999). Bacterial Biofilms: A Common Cause of Persistent Infections. *Science*, *284*(5418), 1318 LP – 1322. https://doi.org/10.1126/science.284.5418.1318

Duneau, D., Ferdy, J.-B., Revah, J., Kondolf, H., Ortiz, G. A., Lazzaro, B. P., & Buchon, N. (2017). Stochastic variation in the initial phase of bacterial infection predicts the probability of survival in D. melanogaster. *ELife*, *6*, e28298. https://doi.org/10.7554/eLife.28298

Ertürk-Hasdemir, D., Broemer, M., Leulier, F., Lane, W. S., Paquette, N., Hwang, D., … Silverman, N. (2009). Two roles for the Drosophila IKK complex in the activation of Relish and the induction of antimicrobial peptide genes. *Proceedings of the National Academy of Sciences*, *106*(24), 9779 LP – 9784. https://doi.org/10.1073/pnas.0812022106

Galac, M. R., & Lazzaro, B. P. (2011). Comparative pathology of bacteria in the genus Providencia to a natural host, Drosophila melanogaster. *Microbes and Infection*, *13*(7), 673–683. https://doi.org/10.1016/j.micinf.2011.02.005

Guzman, L. M., Belin, D., Carson, M. J., & Beckwith, J. (1995). Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *Journal of Bacteriology*, *177*(14), 4121–4130. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/7608087

Khalil, S., Jacobson, E., Chambers, M. C., & Lazzaro, B. P. (2015). Systemic bacterial infection and immune defense phenotypes in Drosophila melanogaster. *Journal of Visualized Experiments : JoVE*, (99), e52613–e52613. https://doi.org/10.3791/52613

Lemaitre, B., & Hoffmann, J. (2007). The Host Defense of Drosophila melanogaster. *Annual Review of Immunology*, *25*(1), 697–743. https://doi.org/10.1146/annurev.immunol.25.022106.141615

Myllymäki, H., Valanne, S., & Rämet, M. (2014). The Drosophila Imd Signaling Pathway. *The Journal of Immunology*, *192*(8), 3455 LP – 3462. https://doi.org/10.4049/jimmunol.1303309

O'Toole, G. A. (2011). Microtiter dish biofilm formation assay. *Journal of Visualized Experiments : JoVE*, (47), 2437. https://doi.org/10.3791/2437

Proctor, R. A., Kriegeskorte, A., Kahl, B. C., Becker, K., Löffler, B., & Peters, G. (2014). Staphylococcus aureus Small Colony Variants (SCVs): a road map for the metabolic pathways involved in persistent infections. *Frontiers in Cellular and Infection Microbiology*, *4*, 99. https://doi.org/10.3389/fcimb.2014.00099

Proctor, R. A., von Eiff, C., Kahl, B. C., Becker, K., McNamara, P., Herrmann, M., & Peters, G. (2006). Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nature Reviews Microbiology*, *4*, 295. Retrieved from https://doi.org/10.1038/nrmicro1384

Santos, V., & Hirshfield, I. (2016). The Physiological and Molecular Characterization of a Small Colony Variant of Escherichia coli and Its Phenotypic Rescue. *PLOS ONE*, *11*(6), e0157578. Retrieved from https://doi.org/10.1371/journal.pone.0157578

Tümmler, B., Weißbrodt, H., Rohde, M., Häußler, S., & Steinmetz, I. (1999). Small-Colony Variants of Pseudomonas aeruginosa in Cystic Fibrosis. *Clinical Infectious Diseases*, *29*(3),

621–625. https://doi.org/10.1086/598644
von Eiff, C., Heilmann, C., Proctor, R. A., Woltz, C., Peters, G., & Götz, F. (1997). A site-directed Staphylococcus aureus hemB mutant is a small-colony variant which persists intracellularly. *Journal of Bacteriology*, *179*(15), 4706–4712. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/9244256
Williamson, R., Collatz, E., & Gutmann, L. (1987). *Mechanisms of action of beta-lactam antibiotics and mechanisms of non-enzymatic resistance*. *Presse médicale (Paris, France : 1983)* (Vol. 15).