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Arthur Menezes April 14, 2020

Pathogen virulence evolution increases pathogen growth in presence of antibiotics

by

Arthur Menezes

Levi Morran
Adviser

Biology

Levi Morran

Adviser

Meleah Hickman

Committee Member

Amanda Freeman

Committee Member

2020

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By

Arthur Menezes

Levi Morran

Adviser

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Abstract

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Serratia marcescens is a human opportunistic pathogen that is capable of rapidly evolving antibiotic resistance, posing a threat to public health. Increases in resistance are often associated with an increase in virulence or a better adaptation to stressful conditions due to the favored co-selection of resistance determinants and virulence modulating genes since they are both carried by mobile genetic elements. It is important to examine how *S. marcescens* evolution can impact its resistance to antibiotics. In order to test how virulence evolution impacts sensitivity to the antibiotic chloramphenicol, we used *S. marcescens* strains from White et al. (2020) that evolved increased virulence against *C. elegans* hosts and determined how antibiotic resistance changed compared to their ancestral strain. While we did not observe any changes to the minimum inhibitory concentration of antibiotic required to suppress bacterial growth, we found a statistically significant increase in *S. marcescens* growth in the presence of chloramphenicol when comparing *S. marcescens* evolved in the presence of hosts to *S. marcescens* evolved in the absence of hosts. Bacterial strains evolved in the presence of two different host genotypes displayed greater growth when compared to the control strains. Most notably, we saw that greater mean change in *S. marcescens* virulence was associated with a greater mean change in growth relative to the ancestor in the presence of chloramphenicol. As *S. marcescens* evolves in the presence of hosts, it becomes more resistant to antibiotics raising concerns as to whether antibiotics will be as effective in the future against evolved strains.

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Introduction

In the 1930's and 1940's, mortality rates due to infection plummeted in many human populations following the emergence of antibiotics (Spellberg, 2014). Soon after Alexander Fleming's discovery of penicillin, he warned that misusing antibiotics would lead to a rise in bacterial resistance to antibiotics (Spellberg, 2014). Now, antibiotic resistance poses a significant problem for public health due to economic, regulatory, and scientific barriers that have contributed to a market failure of new antibiotic research and development (Spellberg, 2014). The economic and regulatory barriers are that development of new antibiotics is seen as a risky, too expensive and not a good investment while scientific barriers are that the "low hanging fruit" antibiotics have already been plucked (Spellberg, 2014). According to the Centers for Disease Control and Prevention, 2.8 million people become infected and 35,000 people die per year due to antibiotic resistant bacteria in the United States. As antibiotic resistance continues to rise, health care workers are left without effective antibiotics to treat infections.

Antibiotics are commonly used to treat infections caused by bacteria in hosts and to eliminate bacteria within the environment (CDC; Ray et al., 2017). *Serratia marcescens* is an environmental gram negative bacterium found in water and soil that causes disease in a wide-range of hosts, including plants, vertebrates and invertebrates (Mahlen, 2011; Iguchi et al., 2014; Marsh & May, 2012). *S. marcescens* is an important pathogen to study as it is a human opportunistic pathogen that is causing a growing concern for public health due to the increase in life threatening *S. marcescens* infections originating in hospitals (Kurz et al., 2003). One of the main dangers is the potential for intrahospital spread and outbreaks specifically in intensive care units and neonatal care units (Mahlen, 2011; Cristina et al., 2019). The first documented

outbreak of hospital acquired *S. marcescens* infections was seen in 1951 when 11 patients at Stanford developed urinary tract infections (Cristina et al., 2019; Mahlen 2011). Although the specific source of an infection is often unidentified, the most common sources of transmission are contaminated hands of healthcare workers and bacterial colonization of catheters (Cristina et al., 2019; Ray et al., 2017). Mortality due to *S. marcescens* infections ranges from 0 to 45%, with many infections causing low rates of mortality due to the pathogen's generally low virulence (Sarvikivi et al., 2004; Messerschmidt et al., 2004; Jang et al., 2001; Cristina et al., 2019). In some cases clinical *S. marcescens* infections can lead to inflammation, fever, sepsis and pneumonia (Rodrigues et al., 2006; Cristina et al., 2019). Treatment options are becoming more limited due to antibiotic resistance. *S. marcescens* is frequently resistant to various antibiotics through plasmid-mediated and chromosomally encoded resistance, posing a threat to public health (Cristina et al., 2019; Mahlen 2011; Rodrigues et al., 2006).

Many *S. marcescens* strains carry plasmid-mediated and chromosomally encoded resistance determinants making them resistant to multiple antibiotics (Mahlen, 2011). The antibiotic chloramphenicol was used to treat *S. marcescens* infections in the 1900s along with other antibiotics aureomycin, streptomycin, kanamycin, and penicillin (Mahlen, 2011). Chloramphenicol is a broad-spectrum antibiotic isolated in 1947 from *Streptomyces venezuelae* that is effective against gram-negative and gram-positive bacteria by inhibiting protein synthesis (Balbi, 2004). When compared to other antibiotics (ceftriaxone, kanamycin, and gentamicin), chloramphenicol was the only one that reduced *S. marcescens* community biomass and viability (Ray et al., 2017).

In a recent experimental evolution project by White et al. (2020) an ancestral strain of *S. marcescens* was passaged through different host populations for 10 generations to examine how host population heterogeneity affects virulence evolution. One common host system used with *S. marcescens* in experimental evolution studies is the nematode *Caenorhabditis elegans. C. elegans* is a free-living nematode with an innate immune system and has been used in the lab for over 40 years (Marsh & May, 2012). *S. marcescens* infects *C. elegans* by colonizing their intestine and eventually causing death (Mallo et al., 2002; Kurz et al., 2003). Most importantly, *C. elegans'* laboratory tractability and their short generation time make them a perfect model organism to use when running long-term evolution within a short time period. With this system, we can examine how the bacteria evolves under different selection regimes after a few passages and hundreds of bacterial generations (Gray & Cutter, 2014). The combination of experimental evolution with *C. elegans* and *S. marcescens* host-pathogen system provides a powerful tool for testing evolutionary theory.

In White et al. (2020), an ancestral strain of *S. marcescens* was passaged through 6 treatment groups to test how host population genotype affects a pathogen's evolutionary trajectory using two *C. elegans* genotypes: ewIR 68 and CB4856. White et al. (2020) saw a 19% increase in mortality when the pathogen was evolved with only ewIR 68 hosts and a 29% increase in mortality when the pathogen was evolved with CB4856 hosts. The increase in pathogen virulence in White et al. (2020) raises questions as to how changes in virulence as pathogens adapt to host populations might affect the pathogen's sensitivity to antibiotics. Recent research has found that increased cooccurrence of antibiotic resistance and virulence factor carriage were strongly linked in *E. coli* populations in a case-controlled study (Zhang et

al., 2015). This poses a growing concern for public health officials as bacterial pathogens evolve greater virulence through contact with hosts and also develop antibiotic resistance, increasing their capacity to evade treatment and containment measures. It is important to examine how *S. marcescens* evolution is impacting its resistance to antibiotics in order to help prevent hospital outbreaks and patient deaths.

Bacterial pathogens that combine multiple antibiotic resistance and high virulence levels are spreading due to the favored co-selection of resistance determinants and virulence modulating genes as they are both carried by mobile genetic elements (Giraud, 2017). Increases in resistance are often associated with an increase in virulence or a better adaptation to stressful conditions (Giraud, 2017). The connection between resistance and virulence of bacterial pathogens has been linked to multidrug resistance (MDR) efflux pumps (Alcalde-Rico et al., 2016). MDR efflux pumps are found in bacterial membranes and are responsible for forcing out antibiotics and host produced antimicrobials from bacterial species (Alcalde-Rico et al., 2016). Efflux pumps can contribute to antibiotic resistance via 3 levels: (1) intrinsic resistance where efflux pumps are present at a basal level of expression, (2) acquired resistance where mutants with high levels of efflux pumps are selected for and (3) phenotypic resistance where overexpression of efflux pumps are triggered by growing conditions or efflux pump effectors (Alcalde-Rico et al., 2016). Although intrinsic and acquired antibiotic resistance can lead to increased *in vivo* bacterial fitness, these fitness costs are dependent on the class of antibiotic and the species of bacteria (Guillard et al., 2016). The environment also has a huge impact in how antibiotic resistance mutations can impact fitness (Guillard et al., 2016). It has been shown that host-produced compounds can trigger the increased expression of MDR efflux

pumps (Rosenberg et al., 2003; Prouty et al., 2004; Alcalde-Rico et al., 2016). Furthermore, the ability of biofilms to tolerate antimicrobials has been linked to an increase in efflux pumps in many organisms including *Candida albicans*, *Pseudomonas aeruginosa,* and *Escherichia coli* (Soto, 2013).

In order to test how virulence evolution impacts sensitivity to chloramphenicol, we used the *S. marcescens* strains from White et al. (2020) that were evolved in no hosts, ewIR 68 hosts, and CB4856 hosts and evolved to become the most virulent and determined the minimum inhibitory concentration (MIC) of chloramphenicol. Then to assess how adaptation to a host affects a pathogen's growth in the presence of antibiotics, we used 13 evolved *S. marcescens* strains from White et al. (2020) that were evolved to no hosts, ewIR 68 hosts, and CB4856 hosts and measured bacterial growth in the presence and absence of chloramphenicol.

Methods

Determining minimum inhibitory concentration (MIC) of chloramphenicol for ancestral and evolved strains of *Serratia marcescens*

For this experiment, we selected the ancestral *S. marcescens* (Sm2170) strain and 4 evolved strains from White et al. (2020) and measured the minimum concentration of chloramphenicol needed to inhibit visible growth. The ancestral strain is responsible for 52% mortality of ewIR 68 *C. elegans* and 45% mortality of CB4856 *C. elegans.* We selected strains evolved in homogenous populations (ewIR 68 or CB4856 *C. elegans* hosts) that showed the highest increase in mortality when infecting its native host (the host it was evolved against). The selected 68 evolved strain was responsible for a 92% mortality of ewIR 68 *C. elegans* while the selected CB evolved strain was responsible for a 83% mortality of CB4856 *C. elegans*. Additionally, we selected two strains from the control treatment (passaged with no hosts for 10 generations) that displayed the highest increase in mortality to both host genotypes (Control 1: 65% ewIR 68 mortality; Control 2: 57% CB4856 mortality). Essentially, we selected the two most virulent strains for each host genotype one evolved in the presence of hosts and one evolved in the absence of hosts.

We streaked the ancestral *S. marcescens* (Sm2170) strain and the evolved strains from White et al. (2020) (stored at -80^{oC} in glycerol) onto one NGM-Lite plate (US Biological, Salem, MA) per strain for a total of 5 plates. After 24 hours of growth at 28^{oC} (standard laboratory procedures), we inoculated 40 colonies from each plate into a 5mL lysogeny broth (LB) test tube and incubated the 5 LB tubes at 28^{oC} for 16 hours, with shaking at 160 rpm.

We selected 40 colonies from each plate into 1 5mL LB tube to account for any genetic variation present on the plate among colonies of *S. marcescens*. After 16 hours, we performed a 1:1000 dilution on each of the 5 bacterial suspensions.

We adapted the broth microdilution protocol from Weigand et al. (2008) to determine the minimum inhibitory concentration of chloramphenicol using 96 well plates. We prepared chloramphenicol concentrations of 128mg/L, 64mg/L, 32mg/L, 16mg/L, 8mg/L, 4mg/L, 2mg/L, 1mg/L, 0.5mg/L and 0.25mg/L in LB. We pipetted 50µL of each chloramphenicol dilution into the respective wells using the layout in Figure 1A. Then we inoculated each well with 50µL of the diluted bacterial suspensions. A growth control (GC) well was used for each strain containing 50µL of LB with no chloramphenicol and 50µL of the diluted bacterial suspension. Lastly, a sterility control (SC) well was used for each strain with 100µL of LB with no bacterial suspension. All strains had two replicates which were completed at the same time.

Once all wells were inoculated, we covered the well plate with a Breathe-Easy© membrane (Diversified Biotech, St. Louis, MO). The well plate was taped in a bin with a wet paper towel and covered with tin foil to prevent evaporation before being incubated at 28^{oC} for 16 hours with shaking at 160 rpm. Growth patterns were interpreted after 20 hours using to determine the minimum inhibitory concentration (MIC) for each strain. Weigand et al. (2008) defined MIC as "the lowest concentration of the antimicrobial agent that inhibits visible growth of the tested isolate as observed with the unaided eye". The lowest concentration of chloramphenicol that produced no visible growth of *S. marcescens*' red pigmentation was defined as the MIC of chloramphenicol for that strain.

Measuring ancestral and evolved strains growth in the presence and absence of chloramphenicol

In this experiment, we selected the ancestral strain, four virulent strains for each host genotype evolved in the presence of that specific host genotype, and five virulent strains evolved in the absence of hosts (control) from White et al. (2020) . The previous MIC experiment selected the most virulent strains while this experiment is using multiple virulent strains. The ancestral strain is responsible for 52% mortality of ewIR 68 and 45% mortality of CB4856. The four 68 evolved strains averaged a 73% mortality of ewIR 68 hosts, the four CB evolved strains averaged a 72% mortality of CB4856 hosts. We also selected five strains evolved in the absence of hosts with increases in mortality when infecting both ewIR 68 and CB4856 hosts. The five control strains averaged a 44% mortality of ewIR 68 hosts and 47% mortality of CB4856 hosts.

We utilized the same methods from the previous MIC experiment to create out 1:000 bacterial suspension. We then used a 96 well plate to test bacterial growth in the presence and absence of chloramphenicol. For the chloramphenicol assay, we combined 50µL of the diluted bacterial suspension and 50µL of 4mg/L in LB of chloramphenicol into each well. This created a final concentration of 2mg/L of chloramphenicol in each well. For the control condition with no chloramphenicol, we combined 50µL of the diluted bacterial suspension and 50µL of LB into each well. We conducted 3 replicates simultaneously for each strain in both assays. Once all wells had been inoculated, we covered the well plates with a breatheeasy membranes. The well plates were taped in a bin with a wet paper towel and covered with tin foil before being incubated at 28^{oC} for 16 hours with shaking at 160 rpm.

After 16 hours, we performed serial dilutions on all wells and plated 100µL of the 10^{-5}, 10^{-6}, and 10^{-7} dilutions onto NGM-Lite plates (US Biological, Salem, MA). Plates were incubated at 28^{oC} overnight for 20 hours. Colonies were counted and the colony forming units (CFU/mL) were calculated for each strain in the presence and absence of chloramphenicol. CFUs were used as a measure of pathogen growth. Only dilutions that yielded plates with 30-400 colonies were counted. CFUs per mL of the original overnight cultures were calculated with the following formula:

$$\frac{CFU}{mL} = \frac{\#\ of\ Colonies \times 10^{*}}{10^{-D}}$$

D= dilution factor plated

*Plating 100µL of a dilution creates an additional 1:10 dilution

After calculating mean CFUs for all strains, we used JMP Pro 15 (SAS, Cary, NC, USA) to run a generalized regression with a negative binomial function to compare mean CFU among all strains in the presence and absence of chloramphenicol. Lastly, we examined the association between changes in pathogen virulence measured by changes in host mortality and mean changes CFUs of evolved pathogen strains compared to the ancestral strain using a linear regression.

Results

In order to test how virulence evolution impacts sensitivity to antibiotics, we determined the minimum inhibitory concentration (MIC) of chloramphenicol for the *S. marcescens* strains from White et al. (2020) that were evolved to no hosts, ewIR 68 hosts, and CB4856 hosts that evolved the highest increased virulence. Increases in virulence were measured by increased in host mortality. Then we selected multiple evolved *S. marcescens* strains also from White et al. (2020) that were evolved to no hosts, ewIR 68 hosts, and CB4856 hosts and measured bacterial growth in the presence and absence of chloramphenicol to assess how adaptation to a host and changes in virulence affects a pathogen's growth.

Ancestral And Evolved Strains Displayed Similar MIC

We hypothesized that the pathogen strains exposed to host populations during the evolution process would exhibit higher MIC of chloramphenicol (lower antibiotic sensitivity) when compared to the pathogen strains not exposed to a host. We based our hypothesis on the idea that the pathogen strain evolved to host populations may have evolved an overexpression of MDR efflux pumps due to the presence of host produced compounds which will help in extruding antibiotics. Accordingly, we expected that the 68 and CB evolved strains would have a higher MIC than the ancestral strain and the control strain which was evolved against no hosts. After analyzing bacterial growth, we determined that all strains had the same MIC of 4mg/L of chloramphenicol which refuted our hypothesis (Figure 1). Visible growth of *S. marcescens*' colonies was seen in well 6 for all strains signifying that 4mg/L of chloramphenicol in well 5 was enough antibiotic to inhibit visible growth.

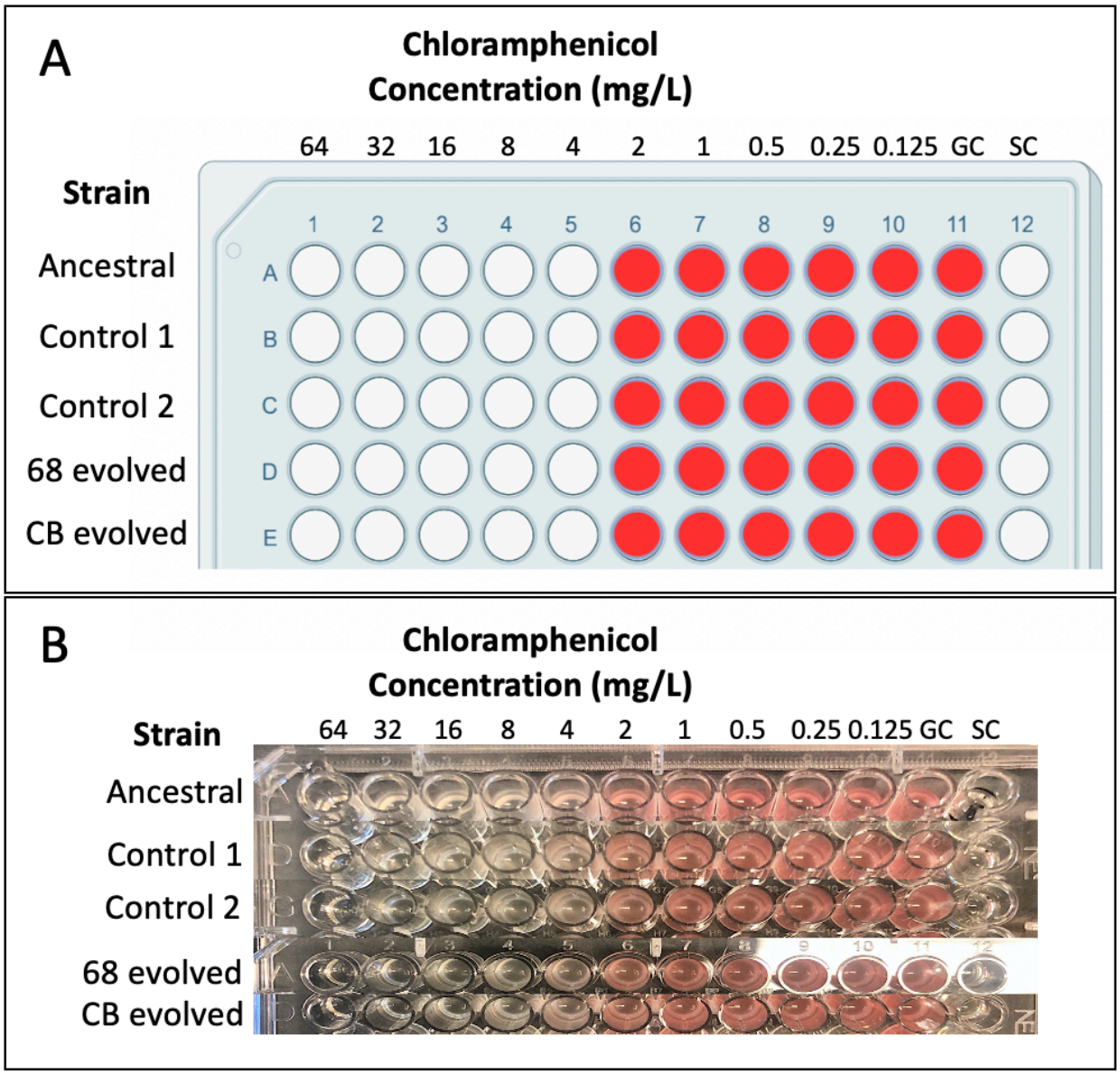

Figure 1. Ancestral and evolved strains exhibit similar MIC of chloramphenicol.
S. marcescens strains evolved in absence of hosts (control 1 and 2) and in the presence of ewIR 68 *C. elegans* (68 evolved) and CB4856 *C. elegans* (CB evolved) all exhibited growth under the same concentrations of chloramphenicol. Despite having evolved greater virulence during the evolution process, the evolved strains displayed the same MIC of 4mg/L of chloramphenicol as the ancestral strain. Red wells display visible *S. marcescens* growth. GC: Growth control well with bacterial suspension and no chloramphenicol. SC: Sterility control with LB and no bacterial suspension.

Evolved Strains Exhibited Greater Growth Than Control Strains In Antibiotics

In order to better assess changes in antibiotic sensitivity that were not captured through the previous MIC experiment, we used 13 evolved *S. marcescens* strains from White et al. (2020) that were either evolved with no hosts, ewIR 68 hosts, or CB4856 hosts and measured bacterial growth in the presence and absence of chloramphenicol. Similar to our previous hypothesis of the effect of MDR efflux pumps on antibiotic removal from the cell, we predicted that strains evolved in the presence of hosts would demonstrate higher CFUs in the presence of chloramphenicol than the control strains that weren't exposed to hosts. We expected higher mean CFUs for the 68 and CB evolved strains when compared to the control and the ancestral strain in the presence of chloramphenicol. We also predicted that all host evolved strains (68 and CB) would have higher growth when compared to the control and ancestral strain in the absence of chloramphenicol based on previous research correlating virulence and growth rates (Chesbro et al., 1969).

Overall, we saw an increase in mean CFUs for all strains in the presence of chloramphenicol when compared to the absence of chloramphenicol (Figure 2). In the presence of chloramphenicol, both 68 and CB evolved strains exhibited similar mean CFUs ($p=0.2955$) (Figure 2). Both 68 and CB evolved strains displayed higher mean CFUs when compared to the control strains ($p=0.0065$ and $p=0.0473$, respectively) which supports our hypothesis that strains evolved in the presence of hosts are less sensitive to antibiotics and therefore exhibit higher CFUs than strains evolved in the absence of hosts (Figure 2). In the absence of chloramphenicol, there were no statistical differences between either evolved strains and the control strains which refutes our hypothesis that more virulent strains would experience higher

CFUs (Figure 2) , which indicates that increased growth was specific to chloramphenicol exposure.

Increased Virulence Is Associated With Increase In Growth In The Presence Of Antibiotics

Lastly, we examined the association between changes in pathogen virulence measured by changes in host mortality and mean changes in growth of evolved pathogen strains compared to the ancestral strain (Figures 3 and 4). Mean change in host mortality was measured using data collected by White et al. (2020). Mean change in growth of evolved *S. marcescens* strains compared to ancestral strain was gathered during this experiment. We examined these two changes to assess the potential for a correlation between the two. We found a significant positive correlation between changes in pathogen virulence and changes in growth in the presence of antibiotics (Figure 3, R^2=0.397, p=0.0210). Thus, the evolution of increased virulence is positively correlated with increased antibiotic resistance. We found no correlation between changes in pathogen virulence and changes in growth in the absence of antibiotics (Figure 4, R^2=0.050, p=0.4633).

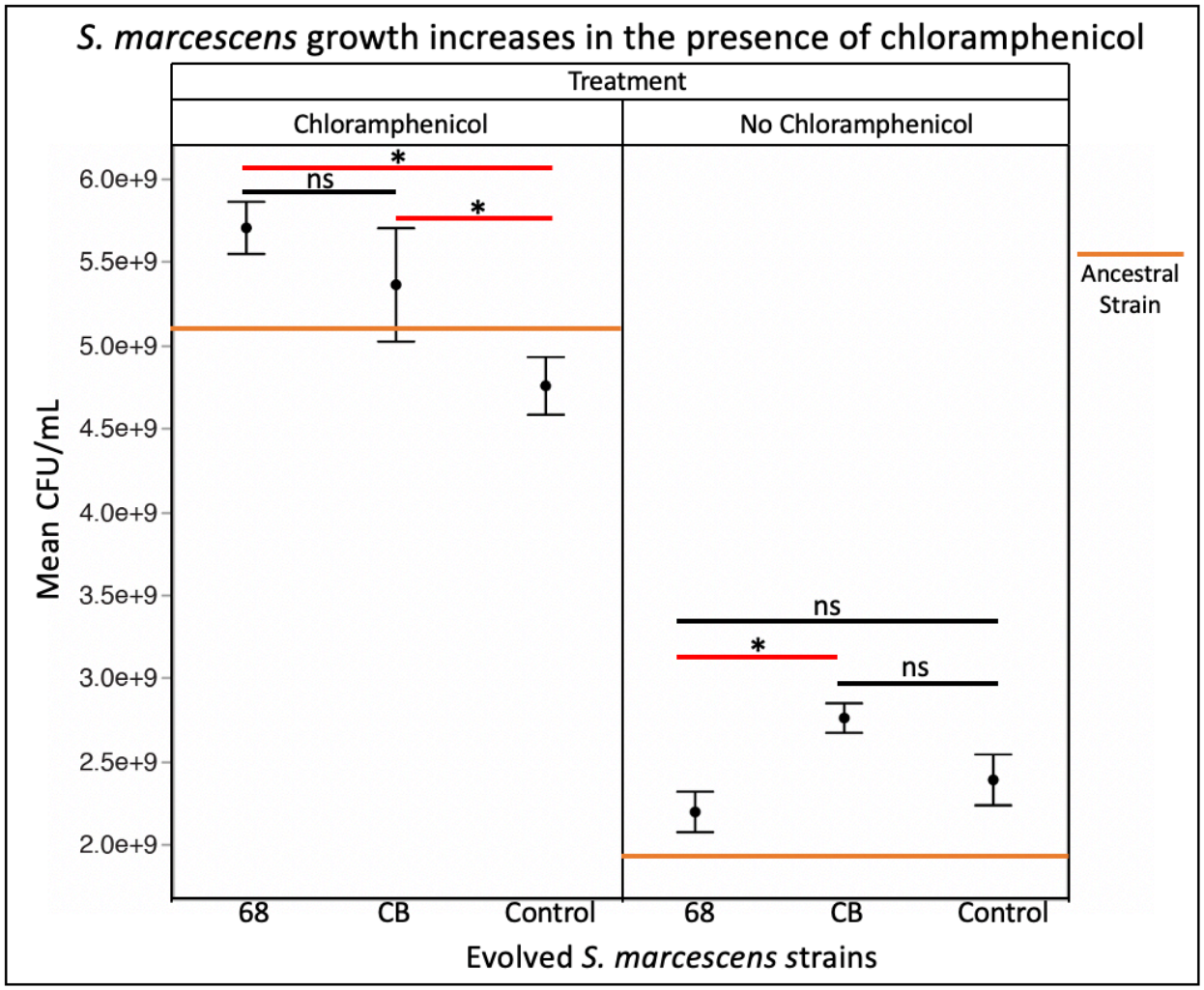

Figure 2. *S. marcescens* growth increases in the presence of chloramphenicol

All three evolved strains displayed increased growth in the presence of chloramphenicol when compared to growth in absence of chloramphenicol. Strains that were evolved to ewIR 68 and CB4856 hosts experienced higher growth than the control in the presence of chloramphenicol ($p=0.0065$ and $p=0.0473$, respectively). In the absence of chloramphenicol, strains evolved to CB4856 hosts experienced higher growth than 68 evolved strains ($p=0.0082$). Error bars display 1 standard error from the mean. Statistically significant ($p<0.05$) differences are displayed with a red line.

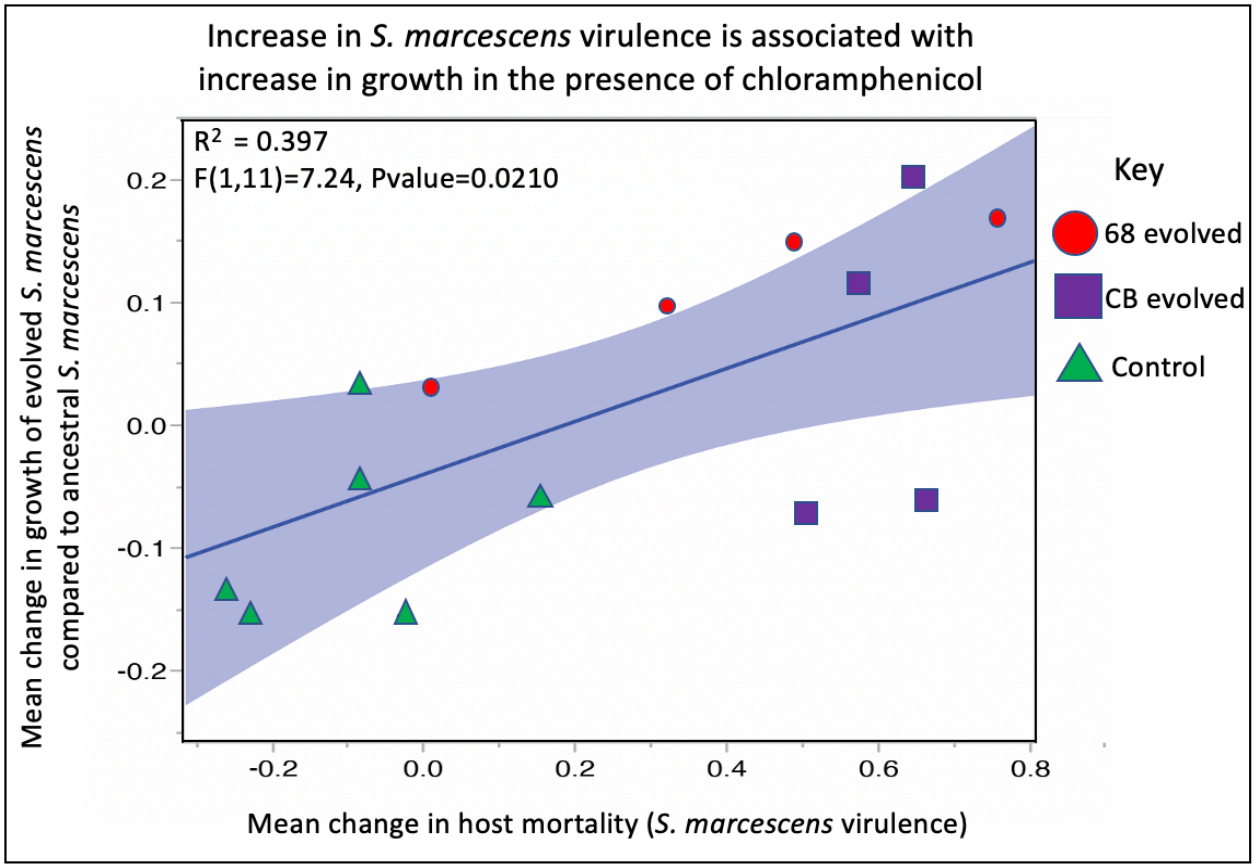

Figure 3. Increase in *S. marcescens* virulence is associated with increase in growth in the presence of chloramphenicol

Mean change in host mortality was measured using data collected by White et al. (2020). Mean change in growth of evolved *S. marcescens* strains compared to ancestral strain in the presence of chloramphenicol was obtained in this study, based on CFUs. We saw a positive correlation between increased pathogen virulence and increased growth in the presence of chloramphenicol, but not in the absence of antibiotics (R^2=0.397, p=0.0210).

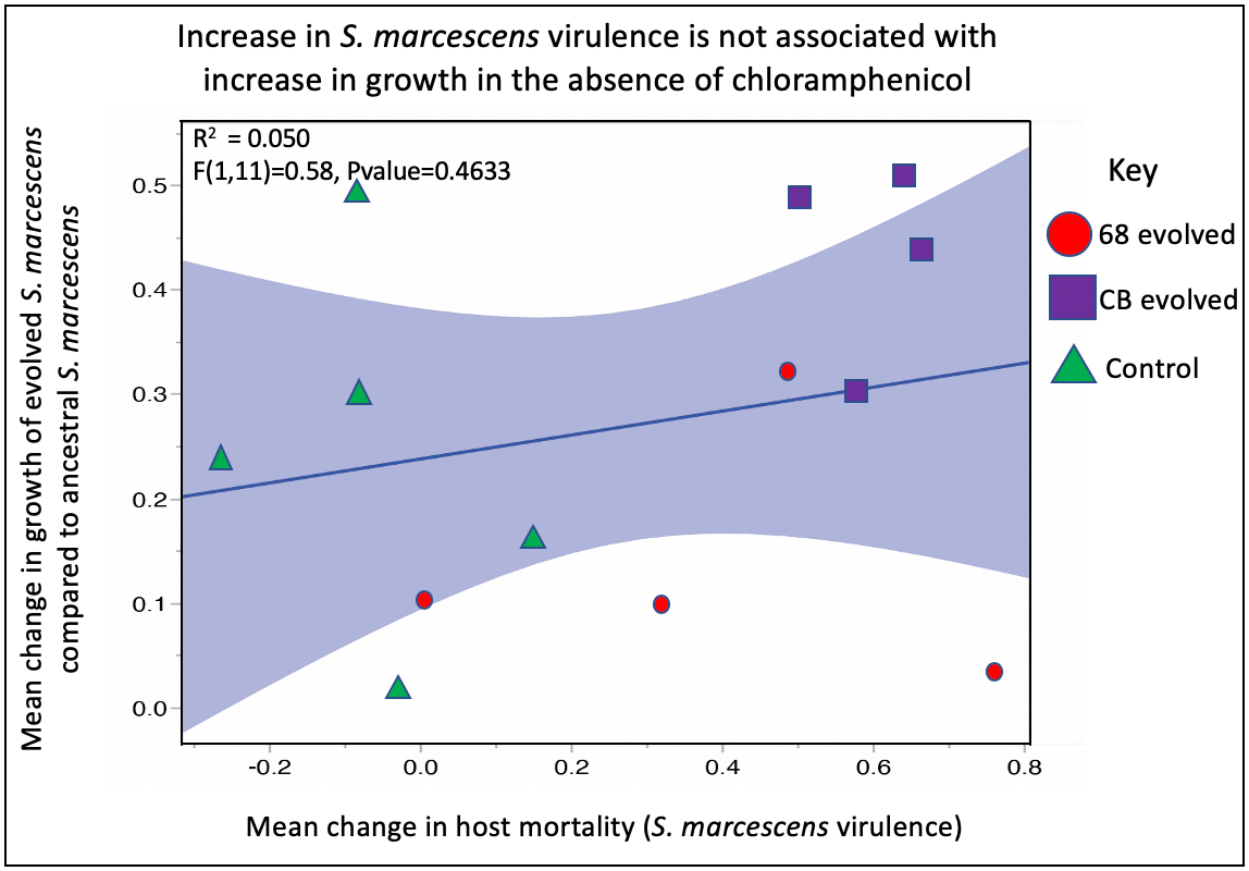

Figure 4. Increase in *S. marcescens* virulence is not associated with increase in growth in the absence of chloramphenicol

Mean change in host mortality was measured using data collected by White et al. (2020). Mean change in growth of evolved *S. marcescens* strains compared to ancestral strain in the absence of chloramphenicol was obtained in this study, based on CFUs. We did not see a positive correlation between increased pathogen virulence and increased growth in the presence of chloramphenicol (R^2=0.050, p=0.4633).

Discussion

We investigated how virulence evolution impacts sensitivity to chloramphenicol by using *S. marcescens* strains evolved in White et al. (2020). We selected strains evolved with no hosts and strains evolved with ewIR 68 hosts or CB4856 hosts that exhibited increased virulence. We were unable to see differences in sensitivity to antibiotics between the ancestral and evolved strains as they all had the same MIC value of 4 mg/L of chloramphenicol (Figure 1). Changes in antibiotic sensitivity at lower concentrations were not ruled out by the inability to see changes in inhibitory MICs and by examining growth visibly which motivated our follow-up experiment to quantitatively examine changes in pathogen growth at antibiotic concentrations less than the MIC value of 4mg/L. We used 13 evolved *S. marcescens* strains from White et al. 2020 that were evolved to no hosts, ewIR 68 hosts and CB4856 hosts and measured bacterial growth in the presence and absence of chloramphenicol to assess how adaptation to a host affects a pathogen's growth (Figure 2). We saw a statistically significant increase in *S. marcescens* growth in the presence of chloramphenicol when comparing *S. marcescens* strains evolved in the presence of hosts to *S. marcescens* evolved in the absence of hosts (Figure 2). Most notably, we saw that greater mean change in *S. marcescens* virulence is associated with a greater mean change in growth relative to the ancestor in the presence of chloramphenicol (Figures 3 and 4).This association causes concern for public health officials who will be left with little to no antibiotics to treat more virulent pathogens as their growth is less sensitive to the impact of antibiotics.

Despite the fact that *S. marcescens* growth in the presence of chloramphenicol visibly looked lighter in color than *S. marcescens* growth in the absence of chloramphenicol, which was

a darker red, we saw an increase in mean CFUs in the presence 2 mg/L of chloramphenicol (Figure 2). The mechanism behind the increase in CFUs in the presence of chloramphenicol remains unknown. However, high growth rates of a different *S. marcescens (*Nima*)* were associated with decreases in prodigiosin concentration, which is responsible for its red pigmentation (Haddix et al., 2008; Haddix and Shanks 2018). Further research is necessary to determine if this association remains true with the *S. marcescens* (Sm2170) strain. It is plausible that antibiotic exposure resulted in loss of prodigiosin production and consequently elevated growth in our *S. marcescens* strain. Nonetheless, further research should be done to examine why we saw an increase in growth in the presence of antibiotics.

In response to our main question of how virulence evolution impacts sensitivity to chloramphenicol, we observed higher growth in the presence of chloramphenicol in strains that evolved increased virulence through experimental evolution in presence of hosts than the control strains (Figure 2). This finding supports our hypothesis that strains evolved in the presence of host populations are less sensitive to antibiotics. We examined the correlation between changes in pathogen virulence and mean changes in growth and observed a positive correlation between changes in pathogen virulence and changes in growth (Figures 3 and 4). Similar associations have been observed in previous research as increases in resistance are often associated with an increase in virulence or a better adaptation to stressful conditions (Giraud, 2017). Bacterial pathogens that combine resistance to multiple antibiotics and high virulence levels are becoming more common to the favored co-selection of resistance determinants and virulence modulating genes (Giraud, 2017). This can cause growing concern for public health due to the increase in life threatening *S. marcescens* infections originating in

hospitals (Kurz et al., 2003). It is possible that the experimental evolution protocol in White et al. (2020) which selected for the most virulent strains to be passaged also selected for *S. marcescens* strains that exhibited an overexpression of MDR efflux pumps caused by the presence of host produced compounds (Rosenberg et al., 2003; Prouty et al., 2004). The connection between resistance and virulence of bacterial pathogens has been linked to multidrug resistance (MDR) efflux pumps which are responsible for extruding antibiotics from the cell (Alcalde-Rico et al., 2016; Giraud et al., 2017). Further research is needed to confirm the exact mechanism by which our host evolved *S. marcescens* strains have higher growth than our control strains in the presence of chloramphenicol.

Overall, it's important to understand how changes in a pathogen's evolutionary trajectory might affect the pathogen's sensitivity to antibiotics since antibiotics are routinely used to treat infections (CDC; Ray et al., 2017). We have shown that strains of *S. marcescens* that were evolved in the presence of host populations are less sensitive to antibiotics. This is a troubling finding that could have major implications for this human opportunistic pathogen in the future (Kurz et al., 2003). It is crucial that health officials are aware of the growing concern as bacterial pathogens evolve greater virulence through contact with hosts and also develop antibiotic resistance increasing their capacity to evade treatment and containment measures.

References

Alcalde-Rico, M., Hernando-Amado, S., Blanco, P., & Martínez, J. L. (2016). Multidrug Efflux Pumps at the Crossroad between Antibiotic Resistance and Bacterial Virulence. *Frontiers in Microbiology*, *7*. doi: 10.3389/fmicb.2016.01483

Balbi, H. J. (2004). Chloramphenicol: A Review. *Pediatrics in Review*, *25*(8), 284–288. doi: 10.1542/pir.25-8-284

Be Antibiotics Aware: Smart Use, Best Care. (2019, November 18). Retrieved from https://www.cdc.gov/features/antibioticuse/index.html

Chesbro, W. R., Wamola, I., & Bartley, C. H. (1969). Correlation of virulence with growth rate in Staphylococcus aureus. *Canadian Journal of Microbiology*, *15*(7), 723–729. doi: 10.1139/m69-127

Cristina, M., Sartini, M., & Spagnolo, A. (2019). Serratia marcescens Infections in Neonatal Intensive Care Units (NICUs). *International Journal of Environmental Research and Public Health*, *16*(4), 610. doi: 10.3390/ijerph16040610

Giraud, E., Rychlik, I., & Cloeckaert, A. (2017). Editorial: Antimicrobial Resistance and Virulence Common Mechanisms. *Frontiers in Microbiology*, *8*. doi: 10.3389/fmicb.2017.00310

Gray, J. C., & Cutter, A. D. (2014). Mainstreaming Caenorhabditis elegans in experimental evolution. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1778), 20133055. doi: 10.1098/rspb.2013.3055

Guillard, T., Pons, S., Roux, D., Pier, G. B., & Skurnik, D. (2016). Antibiotic resistance and virulence: Understanding the link and its consequences for prophylaxis and therapy. *BioEssays*, *38*(7), 682–693. doi: 10.1002/bies.201500180

Haddix, P. L., Jones, S., Patel, P., Burnham, S., Knights, K., Powell, J. N., & Laform, A. (2008). Kinetic Analysis of Growth Rate, ATP, and Pigmentation Suggests an Energy-Spilling Function for the Pigment Prodigiosin of Serratia marcescens. *Journal of Bacteriology*, *190*(22), 7453–7463. doi: 10.1128/jb.00909-08

Haddix, P. L., & Shanks, R. M. Q. (2018). Prodigiosin pigment of Serratia marcescens is associated with increased biomass production. *Archives of Microbiology*, *200*(7), 989–999. doi: 10.1007/s00203-018-1508-0

Iguchi, A., Nagaya, Y., Pradel, E., Ooka, T., Ogura, Y., Katsura, K., … Hayashi, T. (2014). Genome Evolution and Plasticity of Serratia marcescens, an Important Multidrug-Resistant Nosocomial Pathogen. *Genome Biology and Evolution*, *6*(8), 2096–2110. doi: 10.1093/gbe/evu160

Jang, T., Fung, C., Yang, T., Shen, S., Huang, C., & Lee, S. (2001). Use of pulsed-field gel electrophoresis to investigate an outbreak of Serratia marcescens infection in a neonatal intensive care unit. *Journal of Hospital Infection*, *48*(1), 13–19. doi: 10.1053/jhin.2001.0947

Kurz, C., Chauvet, S., Andres, E., Auroze, M., Vallet, I., & Ewbank, J. J. (2003). Virulence factors of the human opportunistic pathogen Serratia marcescens identified by in vivo screening. *The EMBO Journal*, *22*(7), 1451–1460. doi: 10.1093/emboj/cdg159

Mahlen, S. D. (2011). Serratia Infections: from Military Experiments to Current Practice. *Clinical Microbiology Reviews*, *24*(4), 755–791. doi: 10.1128/cmr.00017-11

Mallo, G. V., Kurz, C., Couillault, C., Pujol, N., Granjeaud, S., Kohara, Y., & Ewbank, J. J. (2002). Inducible Antibacterial Defense System in C. elegans. *Current Biology*, *12*(14), 1209–1214. doi: 10.1016/s0960-9822(02)00928-4

Marsh, E. K., & May, R. C. (2012). Caenorhabditis elegans, a Model Organism for Investigating Immunity. *Applied and Environmental Microbiology,78*(7), 2075-2081. doi:10.1128/aem.07486-11

Messerschmidt, A., Prayer, D., Olischar, M., Pollak, A., & Birnbacher, R. (2004). Brain abscesses after Serratia marcescens infection on a neonatal intensive care unit: differences on serial imaging. *Neuroradiology*, *46*(2), 148–152. doi: 10.1007/s00234-003-1140-8

Prouty, A. M., Brodsky, I. E., Falkow, S., and Gunn, J. S. (2004). Bile-salt-mediated induction of antimicrobial and bile resistance in *Salmonella typhimurium*. *Microbiology* 150(Pt. 4), 775–783. doi: 10.1099/mic.0.26769-0

Ray, C., Shenoy, A. T., Orihuela, C. J., & González-Juarbe, N. (2017). Killing of Serratia marcescens biofilms with chloramphenicol. *Annals of Clinical Microbiology and Antimicrobials*, *16*(1). doi: 10.1186/s12941-017-0192-2

Rodrigues, A. P., Holanda, A. R. M., Lustosa, G. P. N., Nóbrega, S. M. B., Santana, W. J., Souza, L. B. S., Coutinho, H. D. M. (2006). Virulence factors and resistance mechanisms of Serratia marcescens. A short review. *Acta Microbiol Immunol Hung,* 53(1), 89-93. doi: 10.1556/AMicr.53.2006.1.6

Rosenberg, E. Y., Bertenthal, D., Nilles, M. L., Bertrand, K. P., and Nikaido, H. (2003). Bile salts and fatty acids induce the expression of *Escherichia coli* AcrAB multidrug efflux pump through their interaction with Rob regulatory protein. *Mol. Microbiol.* 48, 1609–1619. doi: 10.1046/j.1365-2958.2003. 03531.x

Sarvikivi, E., Lyytikäinen, O., Salmenlinna, S., Vuopio-Varkila, J., Luukkainen, P., Tarkka, E., & Saxén, H. (2004). Clustering of Serratia marcescens Infections in a Neonatal Intensive Care Unit. *Infection Control & Hospital Epidemiology*, *25*(9), 723–729. doi: 10.1086/502467

Soto, S. M. (2013). Role of efflux pumps in the antibiotic resistance of bacteria embedded in a biofilm. *Virulence* 4, 223–229. doi: 10.4161/viru.23724

Spellberg, B. (2014). The future of antibiotics. *Critical Care*, *18*(3), 228. doi: 10.1186/cc13948

Wiegand, I., Hilpert, K., & Hancock, R. E. W. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols*, *3*(2), 163–175. doi: 10.1038/nprot.2007.521

White, P. S., Choi, A., Pandey, R., Menezes, A., Penley, M., Gibson, A. K., … Morran, L. (2020). Host heterogeneity mitigates virulence evolution. *Biology Letters*, *16*(1), 20190744. doi: 10.1098/rsbl.2019.0744

Zhang, L., Levy, K., Trueba, G., Cevallos, W., Trostle, J., Foxman, B., … Eisenberg, J. N. S. (2015). Effects of Selection Pressure and Genetic Association on the Relationship between Antibiotic Resistance and Virulence in Escherichia coli. *Antimicrobial Agents and Chemotherapy*, *59*(11), 6733–6740. doi: 10.1128/aac.01094-15