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The coincidental evolution hypothesis proposes that virulence in an opportunistic bacterial pathogen arises in response to selection from predators in the outside-host environment. Opportunistic pathogens, unlike obligate pathogens, do not need a host for survival, and exist in multiple environments where they face selective pressure from eukaryotic predators. This results in a population of bacteria with the best defenses against predation—mechanisms which "coincidentally" cause harm during host infection. Few studies have examined the coincidental evolution hypothesis, and those that have present conflicting results.

Here, we investigated the validity of the coincidental evolution hypothesis by subjecting the opportunistic pathogen *Serratia marcescens* to three different scenarios: (1) co-culture with the ciliate predator *Tetrahymena thermophila*, (2) growth in the absence of predators alone in media, and (3) growth within the guts of germ-free honey bees. We hypothesized that strains evolved in the presence of a predator would display increased virulence and strains evolved in the absence of predators or within a host would exhibit decreased virulence.

Our study revealed that evolution in the presence of a predator resulted in increased virulence of *S. marcescens*. When compared to the media-evolved isolate, all predator-evolved lines exhibited increased pathogenicity in honey bees. However, when investigating how within-host evolution impacts virulence, we observed little to no change in virulence in host-evolved lines. We hypothesize that the lack of attenuated

virulence in within-host-evolved strains is due to lack of selective pressure due to *S. marcescens* already being found at low levels in the bee gut.

Overall, our findings indicate that predation plays a role in the evolution of virulence in opportunistic pathogens and thus support the coincidental evolution hypothesis. We were also able to identify mutations and genes potentially associated with virulence. This study sheds light of the factors that impact virulence and drive the evolution of opportunistic pathogens.

THE COINCIDENTAL EVOLUTION HYPOTHESIS: EXAMINING THE FACTORS

THAT AFFECT VIRULENCE IN AN OPPORTUNISTIC PATHOGEN

by

Heather A. Hopkins

A Thesis Submitted to
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Approved by

____
Committee Chair

APPROVAL PAGE

This thesis written by Heather A. Hopkins has been approved by the following committee of the Faculty of The Graduate School at the University of North Carolina at Greensboro.

Committee Chair ________________________________
Kasie T. Raymann

Committee Members ________________________________
Bryan McLean

____
Gideon Wasserberg

____
Date of Acceptance by Committee

____
Date of Final Oral Examination

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CHAPTER I

INTRODUCTION

1.1 Virulence Maintenance and Evolution

Host/pathogen interactions and the evolutionary arms race that ensues have courted controversy surrounding the evolution and preservation of virulence. Virulence factors—including adhesins, extracellular toxins, and secretion systems—allow pathogens to colonize a host and evade its immune system. While these factors are integral to realizing the pathogen's life history, maintaining them is costly (Casadevall & Pirofski, 2019). Moreover, harming a host makes little evolutionary sense: hosts provide pathogens with necessary nutrients and an ideal environment in which to reproduce. If a pathogen is so virulent that the host dies, so, too, does the pathogen.

The debate surrounding the maintenance of virulence factors has garnered several evolutionary hypotheses to explain their persistence, starting with the avirulence hypothesis. Considered the "conventional wisdom" until the 1980s, the avirulence hypothesis stated that disease will always occur when a novel pathogen infects a host (Adiba et al., 2010). Over time, disease will attenuate and the once-harmful pathogen will become avirulent.

This thinking, however, was flawed. There is little evidence to support that longer established host/pathogen associations result in avirulence (Alizon et al., 2009). Avirulence was thus superseded by evolutionary hypotheses that consider mode

of transmission a major determinant of pathogen virulence (Adiba et al., 2010). The coincidental evolution hypothesis, however, proposes that virulence is determined by adaptation to non-host environments. This hypothesis considers the evolution and maintenance of virulence in opportunistic pathogens, as opposed to the others, which only apply to obligate pathogens.

1.2 Opportunistic vs. Obligate Pathogens

Obligate bacterial pathogens cannot survive without a host, and so must cause a certain degree of virulence in the host in order to be successfully transmitted to others. Opportunistic pathogens (OPs), on the other hand, can live in multiple environments including soil, bodies of water, organic and inorganic surfaces as well as inside hosts. OPs are generally harmless, but as their name suggests can cause disease if given the opportunity. For example, if a host is immunocompromised or suffers from microbiome dysbiosis—an imbalance in the regular makeup of the gut flora (e.g., due to antibiotic use)—they are at an increased risk for contracting infection from an OP (Burke & Lamont, 2014; Martin & Bachman, 2018; Vázquez-Boland et al., 2001). As opposed to obligate pathogens, OPs are rarely transmitted from one host to another. So, if an OP is capable of surviving with or without a host, and if transmission is not a factor in determining its fitness, why are OPs virulent at all?

The factors detailed above are reasons why the coincidental evolution hypothesis (CEH) is currently the only hypothesis that can explain virulence in OPs. The CEH states that OPs, in their natural, outside-host environment, are exposed to selective pressure in the form of predation from eukaryotic microorganisms resulting in the evolution of

predator defense mechanisms that coincidentally act as virulence factors within a host environment. For example, amoebae and protists feed on bacteria, culling the slowest and weakest from the population—leaving those with the best defenses to flourish. If introduced to an immunocompromised host, those same defense mechanisms used to resist predation manifest as virulence factors, "coincidentally" causing disease (Figure 1).

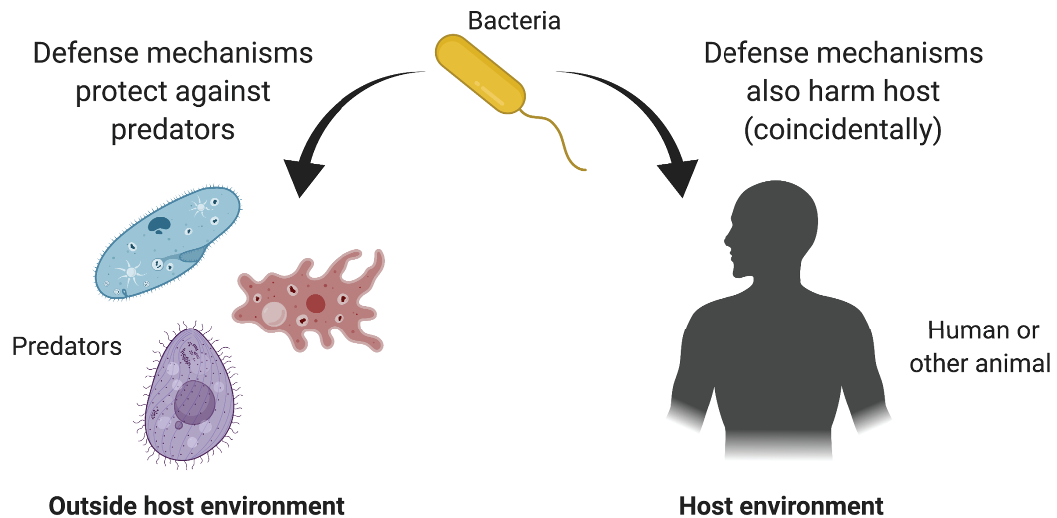

Figure 1. Schematic demonstrating the coincidental evolution hypothesis. Opportunistic bacterial pathogens evolve defense mechanisms against predators in the outside host environment, which act as virulence factors during host infection—coincidentally causing harm.

1.3 Previous Work

Few studies have investigated the CEH, and those that have present conflicting results. In one study, an OP was experimentally evolved in the presence of a eukaryotic predator to determine if predation increased its virulence (Friman et al., 2009). The model systems used in this study included the OP *Serratia marcescens,* the protist

predator *Tetrahymena thermophila*, and the infection model, *Parasemia plantaginis*, a moth species. After evolving the OP with the predator, moth larvae were infected with the ancestral and predator-evolved *S. marcescens* strains. Virulence was attenuated in the predator-evolved strain compared to the ancestral strain as evidenced by decreased mortality in the moth larvae (Friman et al., 2009)—results that disagreed with the CEH (Figure 2).

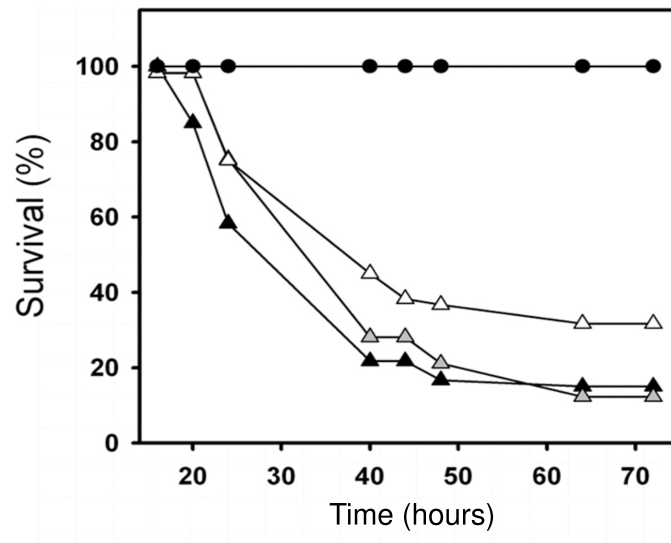

Figure 2. Moth larvae survival (y-axis) after injection with different *S. marcescens* strains. Mortality is increased in the ancestral strain (black triangles) and control (gray triangles) compared to the predator-evolved strain (white triangles). Black circles denote survival of control larvae after injection with sterilized water (Friman et al., 2009).

In another study, human commensal and pathogenic strains of the OP *Escherichia coli* were evolved with the amoebae, *Dictyostelium discoideum*, at different predator:prey population ratios (Adiba et al., 2010). In the natural environment, *D. discoideum* is a predator of *E. coli*. The pathogenic strains—if confined to the human intestinal tract—act as commensals with the host, but cause disease (and sometimes death) if introduced to

sterile environments such as the bloodstream, the cerebrospinal fluid, or the urinary tract (Adiba et al., 2010).

The authors of this study tested if pathogenic strains of *E. coli* were better equipped to withstand predation from *D. discoideum* than commensal strains. While the commensal strains succumbed to the predator at all predator:prey population densities, at certain densities the pathogenic strains were able to resist *D. discoideum*—even sometimes damaging the cell membrane and killing the predator (Adiba et al., 2010). Furthermore, a strong association was found between the ability of the pathogenic strains to resist predation and their ability to kill a mouse infection model—evidence that supports the CEH (Adiba et al., 2010).

In order to determine whether competition, parasitism, and/or predation influence virulence, the OP *S. marcescens* was evolved in the presence of an amoeba, a bacteriophage, and a protist (Zhang et al., 2014). The amoeba, *Acanthamoeba castellanii*, and the protist, *T. thermophila*, are natural predators of *S. marcescens*, whereas the bacteriophage, *Semad11*, is a known virus of *S. marcescens* (Zhang et al., 2014). *S. marcescens* was experimentally evolved with these organisms in all combinations, and then used to infect fruit flies. Every evolved strain showed decreased virulence in a fruit fly infection model compared to the ancestral strain of *S. marcescens*, thus challenging the CEH (Figure 3, Zhang et al., 2014).

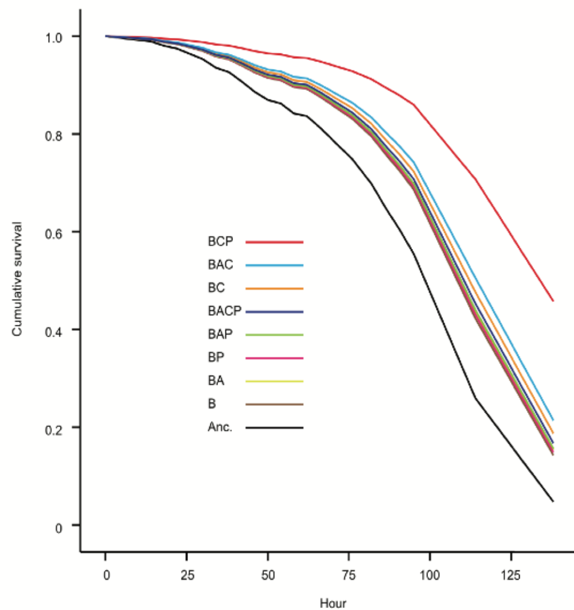

Figure 3. Survival of fruit flies (y-axis) after infection with ancestral or evolved *S. marcescens* strains. All evolved strains (BCP, BAC, BC, BACP, BAP, BP, BA, B) are less virulent than the ancestor (black line) (Zhang et al., 2014).

In another study, *S. marcescens* was again evolved—this time in the *absence* of predators—within the guts of fruit flies and in an artificial, outside-host environment (Mikonranta et al., 2015). The purpose of this study was to determine if removing selection from predators resulted in attenuated virulence. In the within-host experiments, *S. marcescens* was serially passaged from fly to fly through food inoculation. Flies were allowed to feed for 65 hours, after which time mortality was noted and bacterial isolates were saved for the next passage. A total of 10 passaging cycles were performed. The outside-host experiments were essentially the same as the within-host, except flies were omitted.

The authors found that virulence was indeed attenuated in the within-host evolved strains when compared to the ancestral and outside-host evolved strains, indirectly

supporting the CEH (Figure 4, Mikonranta et al., 2015). Little difference in virulence was observed between the ancestor and the outside host strains.

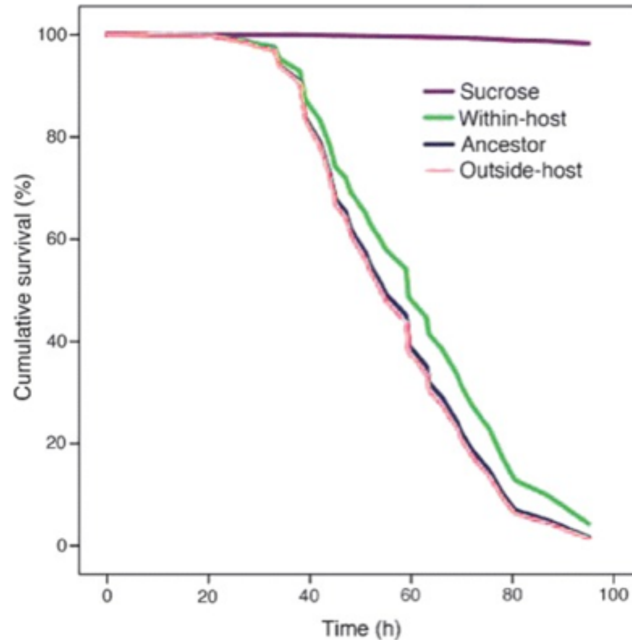

Figure 4. Survival of fruit flies (y-axis) after infection with either the ancestral bacteria (black), the outside-host evolved bacteria (pink), the within-host evolved bacteria (green), or with sucrose (purple). Virulence was reduced in the within-host evolved *S. marcescens* strain (Mikonranta et al., 2015).

1.4 Study Goal

The overarching goal herein was to identify the factors driving the evolution of virulence and the mechanisms responsible for virulence in a ubiquitous opportunistic bacterial pathogen. The scant research that exists illustrates the need for further testing of the CEH, as it is still unclear how predation or lack thereof affect virulence in an OP during host infection. Only one of the referenced studies simultaneously examined both within- and outside-host factors affecting virulence, but it failed to investigate the

influence of predators. Furthermore, none of the studies sequenced the evolved bacterial genomes to confirm that mutations had occurred in the genes responsible for virulence.

This thesis addressed these issues through experimentally evolving a generalist OP (*S. marcescens*) in the presence of a eukaryotic predator, in its absence, or within a host. *S. marcescens* is a Gram-negative bacterium responsible for infecting a wide array of organisms, including plants, fish, insects, and mammals (Mikonranta et al., 2015). In humans, it is responsible for hospital-acquired respiratory, urinary tract, and wound infections, and is a multidrug resistant pathogen (MDR) (Hertle & Schwarz, 2004). It can also cause bacteremia and septicemia, pneumonia, and occasionally even death in the immunocompromised (Haddy et al., 1996; Villari et al., 2001). Once thought to be harmless to the human gastrointestinal tract, recent evidence shows that it damages intestinal epithelial cells (Ochieng et al., 2014).

Serratia marcescens is also an OP of *Apis mellifera*, the honey bee (our host model system). When found at low levels in the honey bee gut, it acts as a commensal (Raymann et al., 2018 mBio). In other circumstances—for example, if there is dysbiosis of the bee gut microbiota due to antibiotic exposure it can become a dangerous pathogen (Raymann et al., 2017). Three strains (KZ2, KZ11, and KZ19), which were isolated from the guts of honey bees have been shown to be lethal to honey bees when administered both orally at high concentrations or when injected into the hemolymph "i.e., insect bloodstream" (Figure 5, Raymann & Moran, 2018). Moreover, these strains share many common virulence factors with virtually all other *S. marcescens* strains—including

chitinases, DNases, gelatinases, hemolysins, proteases, siderophores, and swarm and swim motility (Raymann et al., 2018).

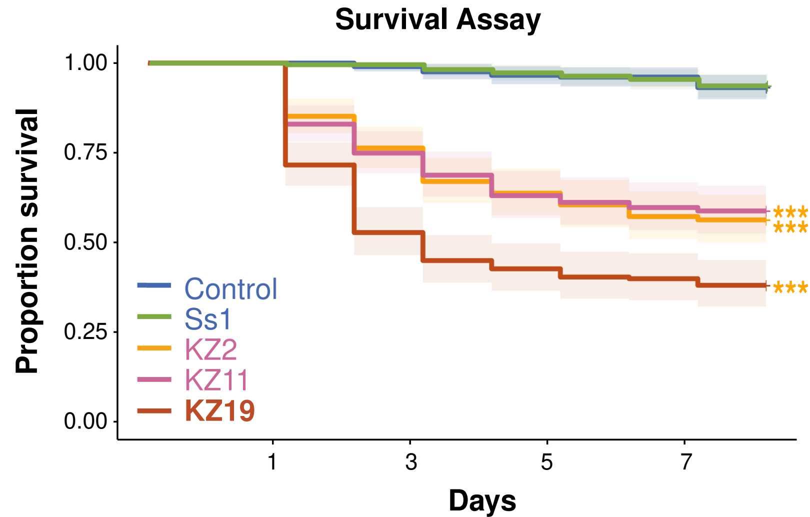

Figure 5. Survival of honey bees (y-axis) after oral exposure to three *S. marcescens* strains (KZ2, KZ11, and KZ19) that were isolated from the guts of honey bees. All three were shown to be pathogenic to honey bees, causing a significant increase in mortality after exposure (Raymann et al., 2018).

The work described here utilizes the honey bee as a model system. Honey bees make for an ideal infection model due to the ability to generate large sample sizes for repeat testing. Honey bees acquire their gut microbiome primarily through oral trophallaxis (when honey bees pass regurgitated nectar between themselves) and coprophagy (the consumption of feces) after emergence (Powell et al., 2014). Thus, if bees are aseptically removed from the cells as pupae—before eclosure occurs—and then incubated under sterile conditions, they can be utilized as "germ-free" models. Additionally, as stated above, *S. marcescens* is an opportunistic pathogen of honey bees, so we can investigate the virulence of this ubiquitous OP using honey bees as a model system.

Understanding how virulence evolves in OPs is a logical first step in combating diseases caused by MDR pathogens such as *S. marcescens*. With this information, new treatments could be developed that reduce hospital-acquired infections caused by OPs, which are increasingly resistant to current antibiotics. This thesis has three objectives:

- **Objective 1**: Determine if the presence or absence of a predator impacts virulence of an OP
- **Objective 2**: Investigate how within-host evolution impacts OP virulence
- **Objective 3**: Sequence genomes to identify virulence genes in an OP

1.5 Objective 1: Determine if the presence or absence of a predator impacts virulence of an OP

The first objective was to determine how the presence or absence of a predator impacts the virulence of OPs. Since virulence factors expressed during infection have been associated with defense mechanisms employed against predation (Rehfuss et al., 2011), we hypothesized that evolution in the presence of a predator would select for defense mechanisms that aid in survival against predation and these mechanisms would then coincidentally increase virulence in the host environment. We also hypothesized that if *S. marcescens* is evolved alone in growth media away from predatory selection, then the pressure to maintain costly virulence factors would be removed (Casadevall & Pirofski, 2019) and virulence would be attenuated during host infection.

To address this objective, we serially passaged *S. marcescens* strain KZ19 (isolated from honey bees) in media with the predator *T. thermophila* or alone in media. *T. thermophila* is a protist found in soil and temperate freshwater environments such as

lakes and ponds. It feeds on bacteria, including *S. marcescens*, and has been used previously as a model organism in other experimental evolution studies (Cairns et al., 2019; Friman et al., 2009; Zhang et al., 2014), making it an ideal predator for this research. After evolving *S. marcescens* in the presence or absence of *T. thermophila*, we evaluated virulence of the evolved strains in honey bees.

1.6 Objective 2: Investigate how within-host evolution impacts OP virulence

The second objective was to determine how within-host evolution impacts the virulence of OPs. Our hypothesis was that if *S. marcescens* is evolved solely within the guts of germ-free honey bees (away from predatory and other outside-host influences), then virulence would be attenuated in a honey bee infection model. Again, genes expressed during infection have been associated with defense mechanisms (Rehfuss et al., 2011), and costly virulence factors should be lost without selective pressure from predators (Casadevall & Pirofski, 2019). So, serially passaging only the OP in a germ-free host should remove selective pressure from predators and thus curtail the need to maintain defense mechanisms (i.e., virulence factors). For this objective, we serially passaged *S. marcescens* strain KZ19 in the guts of germ-free honey bees, and then tested the virulence of the evolved strains in honey bees.

1.7 Objective 3: Sequence genomes to identify virulence genes in an OP

The third and final objective of this thesis was to identify the genes associated with OP virulence. Adaptation to new environments and selective pressures occurs rapidly for organisms with small, streamlined genome sizes such as bacteria. Thus, we hypothesized that if an OP is consistently in the presence of a predator, mutations that aid

in resistance to predation should arise in the genome and potentially become fixed in the population via positive selection. On the other hand, OPs that never meet a predator have no need to maintain these costly defense/virulence mechanisms. Furthermore, if an OP is limited to the host environment, harming the host provides it no benefit. Therefore, genes associated with defense/virulence in media-evolved and host-evolved strains will not be under positive selection, and mutations should accumulate—resulting in function loss and eventually loss of the gene(s). For this objective, we sequenced the genomes of all of the evolved *S. marcescens* strains and identified mutations associated with gain and loss of virulence. Sequencing of the evolved genomes also allowed us to confirm underlying causes of virulence and also determine how quickly bacteria adapt under different selective pressures.

CHAPTER II

METHODS

2.1 Experimental Design for Objective 1

In order to determine how predator presence impacts the virulence of OPs, *S. marcescens* strain KZ19 was evolved in three separate culture flasks with *T. thermophila* for 60 days. *T. thermophila* was grown in 5mL Neff media, a media made specifically for culturing *Tetrahymena* (Cassidy-Hanley, 2012), for three days, at which time 21mL from several cultures were combined and mixed gently. This was performed to ensure that each flask received approximately the same amount of *T. thermophila*. Three milliliters of the combined culture were pipetted into each of the four culture flasks (*T. thermophila* control plus the three treatment flasks). Bacteria were also grown in Neff media, and an initial prey density of 10^2 bacterial cells/mL were added to each treatment flask. This density allowed for the maintenance of both organisms when cultured together.

To control for coevolution in the predator species, 10uL of bacteria were collected daily from the treatment culture flasks and exposed to new, axenic cultures of *T. thermophila*. Luria-Bertani (LB) agar plates were loop inoculated from the culture flasks each day and checked 24 hours later to confirm bacterial growth. The flasks were also checked under a microscope daily to ensure that *T. thermophila* was still alive and to assess the population density. See Figure 6 for experimental setup.

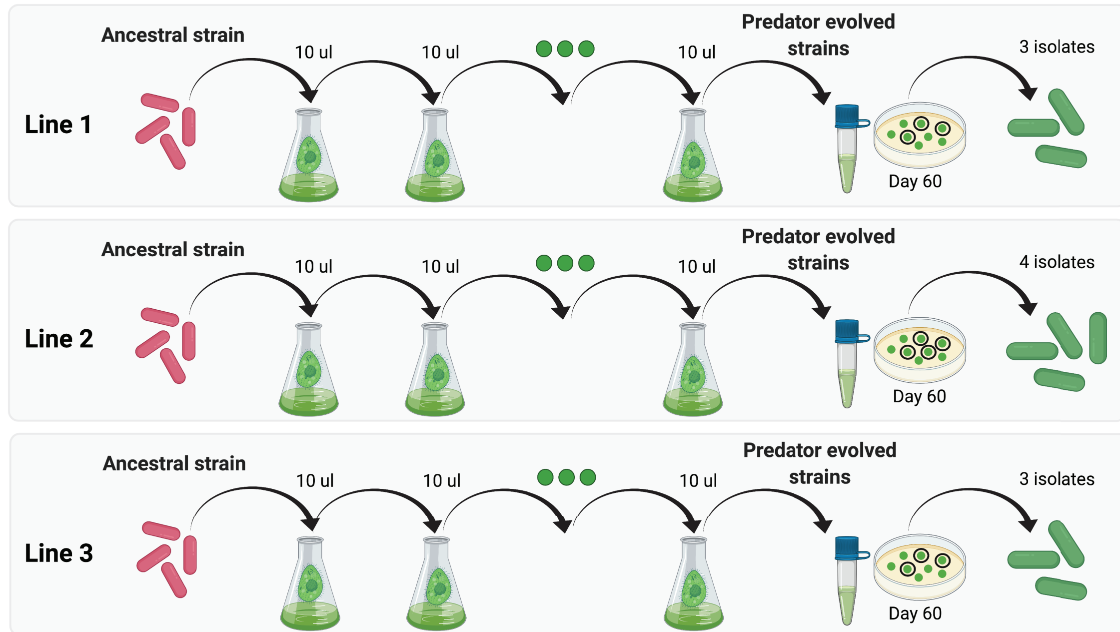

Figure 6. Experimental design for predator-evolved lines. *S. marcescens* strain KZ19 was evolved in the presence of the ciliate predator, *T. thermophila* in triplicate (three lines) for 60 days. After 60 days, ~10 ul of the evolved cultures were plated on LB agar. After 24 hours, isolates were randomly picked and pure cultured for sequencing and virulence assays. The remaining cultures were preserved in 20% glycerol at -80C.

In order to determine how the absence of predation impacts the virulence of OPs, *S. marcescens* strain KZ19 was evolved alone in three separate culture flasks for 60 days. Cultures were prepared with a starting population density of 10^2 bacterial cells/mL in Neff media (Cassidy-Hanley, 2012). In order to mirror the experimental design of the predator exposed evolution, 10 microliters of bacteria were collected from the culture flasks at 24-hour intervals and exposed to clean media. LB agar plates were loop inoculated from the culture flasks each day and checked 24 hours later to confirm bacterial growth. See Figure 7 for experimental setup.

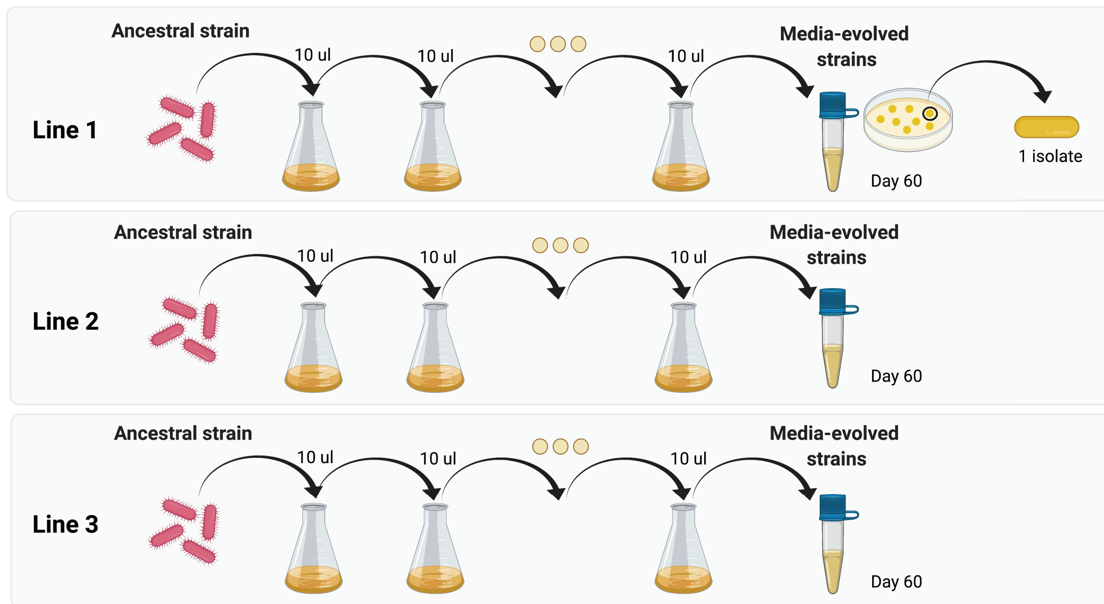

Figure 7. Experimental design for media-evolved lines. *S. marcescens* strain KZ19 was evolved away from predators, alone in growth media in triplicate for 60 days. After 60 days, ~10ul of the evolved line 1 culture were plated on LB agar. After 24 hours, one isolate was randomly picked and pure cultured for sequencing and virulence assays. The remaining cultures were preserved in 20% glycerol at -80C.

After 60 days, 800uL of *T. thermophila* and *S. marcescens* co-cultures (predator-evolved) and the *S. marcescens* mono-cultures (media-evolved) were taken from each of the culture flasks—200uL of which were pipetted into four different cryogenic storage tubes (12 total) containing 800uL of 20% glycerol. These tubes were vortexed and then frozen at -80°C (*T. thermophila* do not survive freezing). Three LB agar plates were also isolation streak inoculated from each of the three culture flasks for both the predator-evolved and media-evolved lines, then incubated at 30°C (Figures 6 and 7). After 24 hours, 10 bacterial colonies were randomly chosen from the LB plates. For the predator-evolved lines three colonies were taken from plate 1, four from plate 2, and three from plate 3 (Figure 6). For the media-evolved lines three colonies were taken from plate 1, four from plate 2, and three from plate 3 (Figure 7). All isolated colonies were inoculated

into 5mL LB broth and incubated at 30°C for 48 hours to create pure cultures. After 48 hours, 200uL from each pure culture were pipetted into cryogenic storage tubes containing 1.6mL 20% glycerol, vortexed, and frozen at -80°C to be later used for virulence assays and sequencing.

Because 10uL of bacteria were passaged each day in our experiments instead of a single clone, we isolated multiple colonies from each line to account for multiple strains that could have arisen in the populations during the experiment. However, it must be noted that the isolates obtained from the same evolved line (flask) could be clones rather than different strains within the population. This same disclaimer also applies to the experimental design for Objective 2.

2.2 Experimental Design for Objective 2

In order to determine how within-host evolution impacts the virulence of OPs, *S. marcescens* was evolved by serially passaging it in germ-free honey bee hosts for 60 days. To begin, five germ-free bees were fed a 0.5 OD sugar syrup solution inoculated with KZ19 media-evolved strain from Objective 2 via the immersion method. This method ensures that each bee ingests the bacteria, because bees will immediately clean themselves and each other after being coated in the sugar solution (Raymann & Moran, 2018). Colonization of *S. marcescens* occurs in the bee within 24 hours (Raymann et al., 2018), thus the guts of each bee were extracted 24 hours after inoculation. The five extracted guts were separately homogenized with 50uL sterile sugar syrup, and then 5uL was fed to 10 new germ-free bees (10 bees per gut, 50 bees total). Bees were kept in cup

cages (five total) in groups of 10 in a climate-controlled environment that mimicked hive conditions: 95% humidity and 35°C.

Over the next 59 days, every 24 hours one bee gut from each of the five cup cages was extracted, homogenized with 50uL sterile sugar syrup, and then 5uL of this was fed to a new group of 10 germ-free bees (10 bees per cup, 50 bees total). Each extracted gut was also inoculated onto LB agar after homogenization, incubated for 24 hours at 30°C, and then checked to confirm that bacteria were present. See Figure 8 for experimental setup.

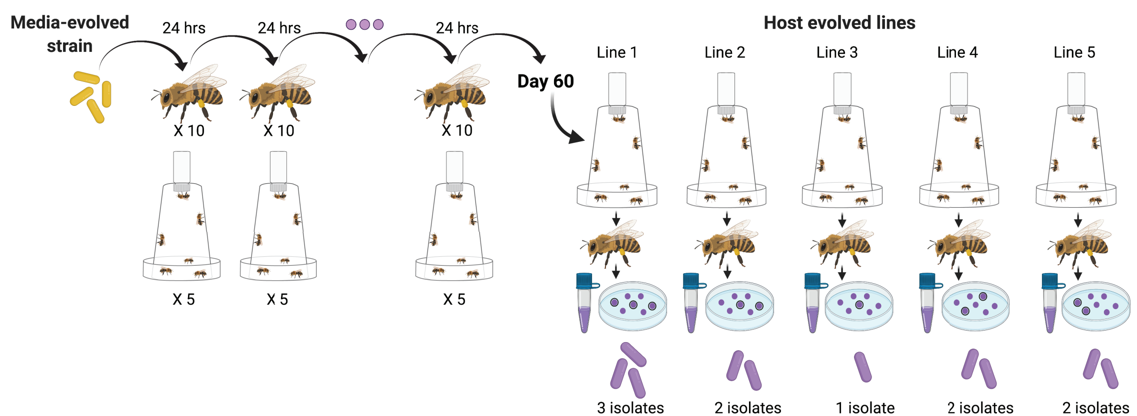

Figure 8. Experimental design for Objective 2. *S. marcescens* media-evolved strain KZ19 was evolved within the guts of germ-free honey bees. Five replicate lines were performed with 10 bees per replicate. After 60 days, one bee was randomly chosen from each line. The guts of one bee from each line were extracted and homogenized and ~10 ul of the gut homogenates were plated on LB agar. After 24 hours, isolates were randomly picked and pure cultured for sequencing and virulence assays. The remaining cultures were preserved in 20% glycerol at -80C.

Once weekly, a PCR using *Serratia*-specific primers was performed on the extracted bee guts to ensure that *S. marcescens* was successfully passaged. Once *Serratia* was confirmed, isolates were grown at 30°C incubation in test tubes containing 3mL LB broth for 24 hours and 200uL from each test tube were preserved in 800uL of 20%

glycerol in cryogenic tubes, vortexed, and frozen at -80°C. If a contaminant was discovered after PCR, the experiment was continued using the saved isolates from the last successful passage rather than restarting the experiment from scratch.

After 60 days, five LB agar plates were isolation streak inoculated with homogenized bee guts (one bee from each cup cage). After 24 hours incubation at 30°C, 10 bacterial colonies were randomly chosen from each LB plate (three from plate 1, two from plate 2, one from plate 3, two from plate 4, and two from plate 5) and incubated at 30°C in 5mL LB broth to create pure cultures (Figure 8). After 48 hours, 200uL from each pure culture were pipetted into cryogenic storage tubes containing 800uL 20% glycerol, vortexed, and frozen at -80°C to be later used for virulence assays and sequencing.

2.3 Virulence Assays

To determine if virulence had changed in predator-, media-, and/or within-host evolved strains, adult honey bee workers were randomly sampled from a colony and given one of four treatments: immersion in a sugar syrup solution inoculated with either 1) the media-evolved strain (one single isolate), 2) a predator-evolved strain (10 total isolates), 3) a host-evolved strain (10 total isolates), or 4) no bacteria (sterile sugar syrup) via the immersion method described in Objective 3. See Figure 9 for experimental setup.

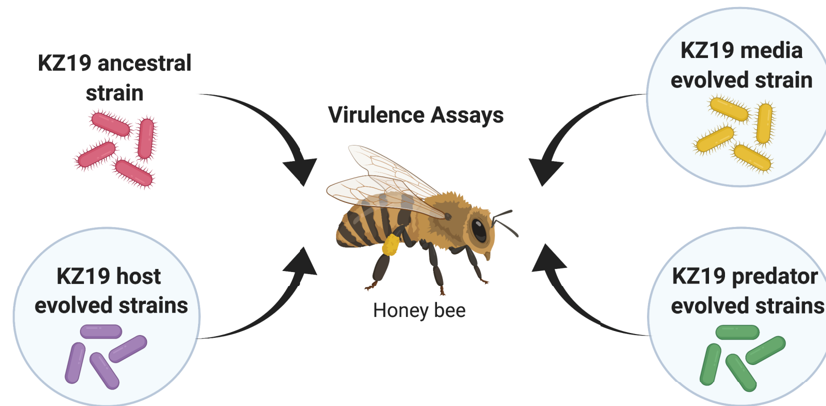

Figure 9. Schematic representing the virulence assays performed for Objectives 1 and 2. Adult honey bee workers were fed either sterile sugar syrup (the control), the ancestral strain, a media-evolved strain, or a predator-evolved strain to determine if experimental evolution increased or decreased virulence. Three to four replicate survival assays were performed for each strain with 100 bees per strain per assay plus 100 control bees.

Once the treatments were administered, bees were kept in cup cages that mimicked hive conditions as detailed in Objective 3. Twenty bees were kept in each cup cage, with five replicate cup cages per treatment—including five for the control, five for the media-evolved strain, five for each predator-evolved isolate, and five for each host-evolved isolate, resulting in one hundred bees per treatment. Each bee was treated with approximately 10 microliters of a 1 OD bacteria-sugar syrup solution, which they ingested while grooming themselves. Over a period of five days, mortality was monitored and recorded. This time interval was chosen because mortality following *S. marcescens* infection occurs primarily within the first five days post-exposure (Raymann et al., 2018). Four replicate assays were done comparing the 10 predator-evolved KZ19 strains to the KZ19 media-evolved strain and to the control (with the exception of evolved strains PE KZ19 3-2 and PE KZ19 3-3, in which three replicates were performed) (Supplemental Figure A1). Three replicate assays were done comparing the

10 KZ19 within-host evolved strains to the KZ19 media-evolved strain (in this case this is the ancestral strain) and to the control (Supplemental Figure A2). Kaplan Meier survival curves were generated for each individual assay performed (Supplemental Figures A1 and A2). All replicate virulence assays were then combined to generate a single survival curve for each evolved isolate (Figures 11 and 12). Survival curves and statistical analyses (Mantel-Cox Log-rank tests and Gehan-Breslow-Wilcoxon tests) were done using GraphPad Prism version 9.1.0.

2.4 Experimental Design for Objective 3

In order to identify the genes responsible for increased or decreased virulence in the predator-, media-, host-evolved and ancestral strains, the genomes of each were sequenced. All were mapped to the KZ19 genome that was sequenced previously (Raymann et al., 2018). See Figure 10 for experimental setup.

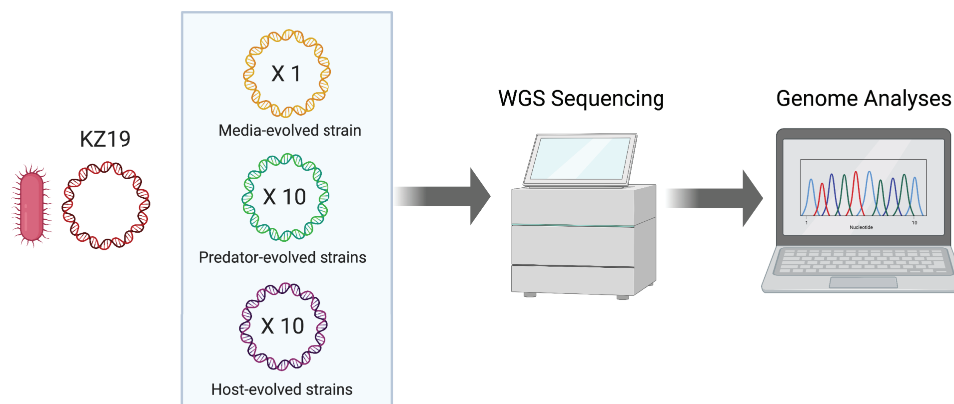

Figure 10. Experimental design for Objective 3. The genomes of 21 evolved strains as well as the ancestral strain were sequenced to determine what mutations had arisen during experimental evolution.

For genome sequencing, pure cultures of the evolved strains and the ancestral strain (22 total) were diluted to ~1 OD at 600nm, which corresponds to ~10^9 cells/mL.

DNA was extracted using the Zymo Quick-DNA Fungal/Bacterial Miniprep Kit (D6005). The DNA was then prepared for whole genome shotgun (WGS) sequencing using the Illumina Nextera DNA Flex Library Prep Kit (20018704). Genome sequencing allowed us to identify mutations in the evolved strains in comparison to the ancestor. The genomes were sequenced on the lab's Illumina iSeq 100. Once sequenced, the samples were demultiplexed using the Illumina BaseSpace Sequence Hub. Raw sequencing reads were trimmed using BBDuk (sourceforge.net/projects/bbmap/) implemented in Geneious Prime, using default settings. Trimmed reads were then assembled by mapping to the ancestral genome in Geneious Prime using Bowtie 2 (Langmead & Salzberg, 2012) with default settings. Average genome coverage for each strain was between 30-50X. Assembled contigs were annotated using RAST (Aziz et al., 2008). For identifying single nucleotide polymorphisms (SNPs), or other mutations, raw sequencing reads were trimmed using Trimmomatic (Bolger et al., 2014) and then BreSeq (Deatherage & Barrick, 2014) was used to identify nucleotide differences between the evolved strains and the ancestral and media-evolved strains (Tables A1 and A2). To confirm that the mutations were not artifacts of using consensus genomes for mapping and SNP calling, all reads of the media-evolved and ancestral strains were also analyzed using BreSeq (Deatherage & Barrick, 2014). Mutations detected in BreSeq were also manually confirmed by visualizing the assemblies and performing SNP detection in Geneious Prime. Our threshold for calling a SNP was that it had to be represented by 90% or more of the mapped reads.

CHAPTER III

RESULTS

3.1 Strain Virulence After Experimental Evolution

Virulence assays comparing the predator-evolved strains to the media-evolved strain showed an increase in virulence in adult honey bees. Bees exposed to the KZ19 predator-evolved strains exhibited decreased survival over five days compared to those exposed to the control or the media-evolved strain. All KZ19 predator-evolved isolates from all three lines were significantly more virulent than the media-evolved strain (Figure 11, Supplemental Figure A1). Predator-evolved (PE) kz19 isolates from lines 1 and 2, and the line 3 isolate PE 3-1 were the most virulent, with mortality around 45% and $P<0.0001$ for both the Mantel-Cox Log-rank and Gehan-Breslow-Wilcoxon tests.

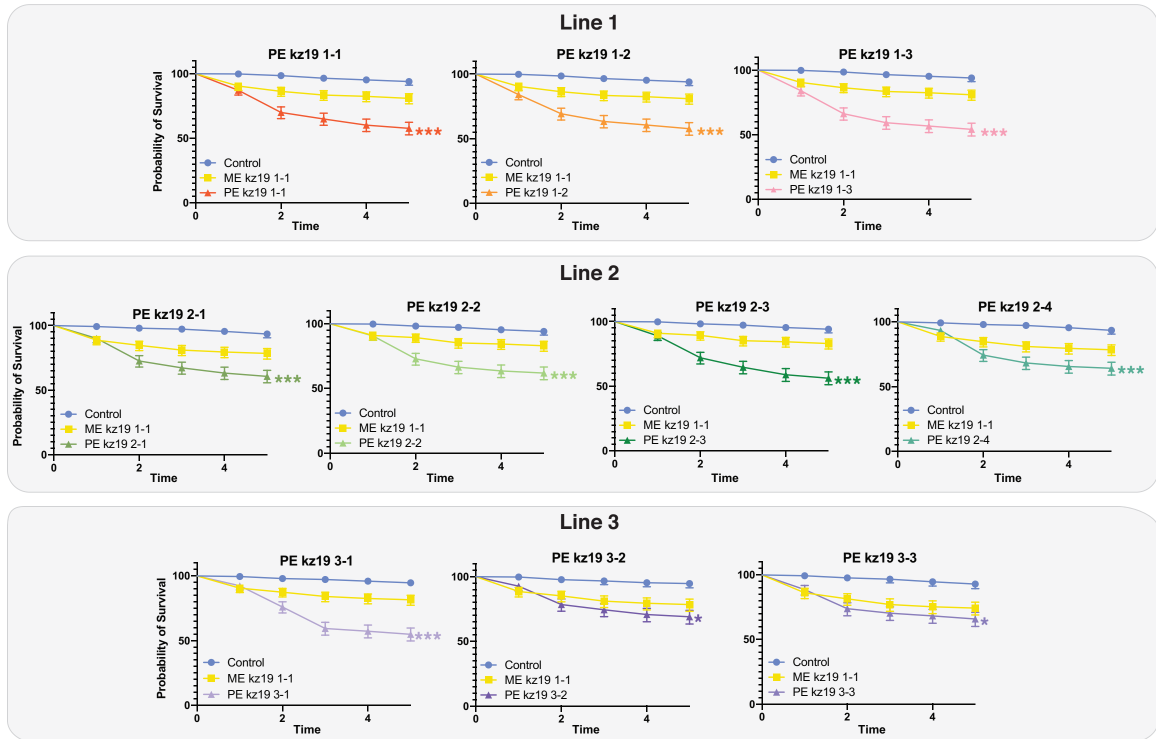

Figure 11. Average probability of survival (y-axis) of honey bees for Objective 1. Bees fed predator-evolved (PE) kz19 isolates showed an increase in mortality over five days compared to the control (blue line) or the media-evolved (ME) kz19 isolate (yellow line). Survival curves were created in GraphPad Prism. Statistical testing was performed using the Mantel-Cox Log-rank test and the Gehan-Breslow-Wilcoxon test. * = $P<0.01$, ** = $P<0.001$, *** = $P<0.0001$ for both tests.

Virulence assays comparing KZ19 host-evolved (HE) strains to the KZ19 media-evolved (ME) strain showed an increase in virulence in adult honey bees in four isolates (one from line 1, two from line 2, and one from line 4), and no significant change in virulence in the six others isolates (Figure 12, Supplemental Figure A2).

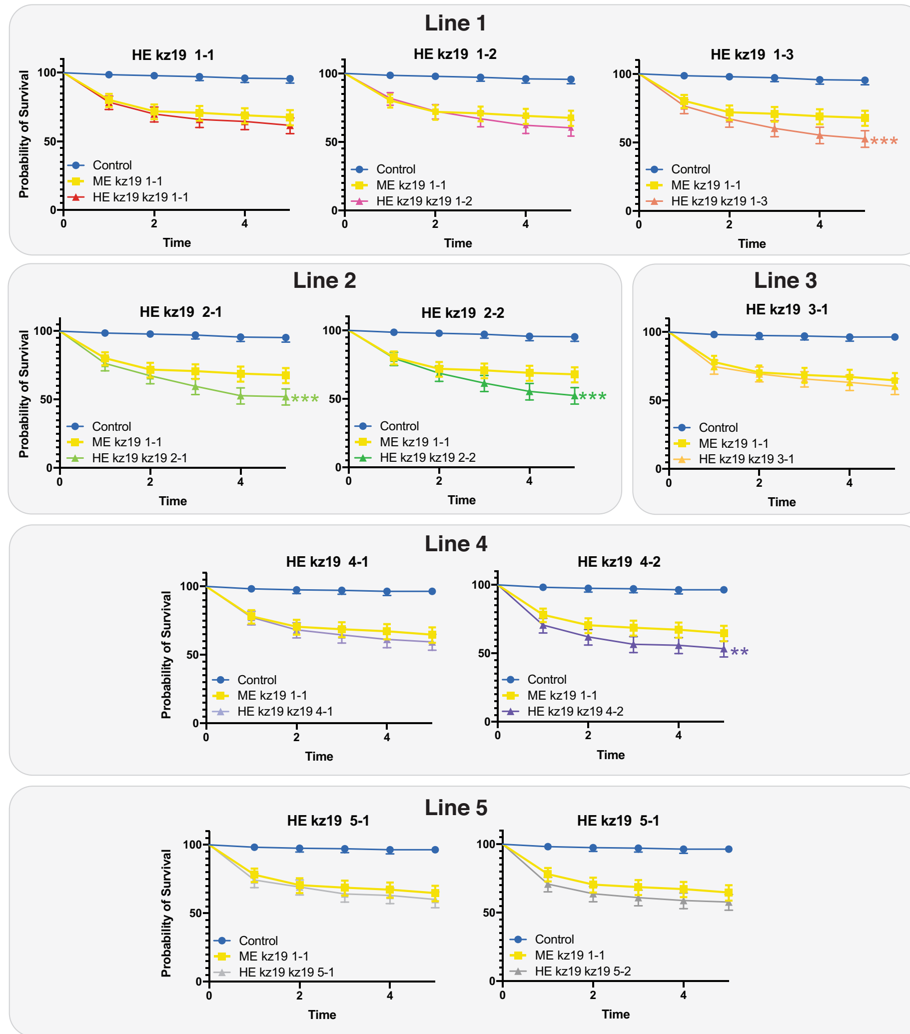

Figure 12. Average probability of survival (y-axis) of honey bees for Objective 2. Bees fed host-evolved (HE) kz19 isolates showed a slight increase in mortality over five days compared to the control (blue line) or the media-evolved (ME) kz19 isolate (yellow line). Survival curves were created in GraphPad Prism. Statistical testing was performed using the Mantel-Cox Log-rank test and the Gehan-Breslow-Wilcoxon test. * = $P<0.01$, ** = $P<0.001$, *** = $P<0.0001$ for both tests.

3.2 Mutations in predator- and media-evolved strains

Sequencing the genomes of the predator- and media-evolved lines allowed us to identify a number of mutations (Table 1). At least one mutation was discovered in all evolved lines and included mutations in both intergenic and coding regions. Note, if the

same mutation was found in multiple isolates from the same line, we only counted it as one mutation since the isolates are likely clones.

Line	Isolate	Contig	Position	Mutation	Type	Description	Annotation
1	ME 1 (isolate 1)	13	457,504	Δ	NS	Δ66 bp	DNA-binding capsular synthesis response regulator RcsB
1	ME 1 (isolate 1)	3	269,652	G→A	NS	CAA→TAA	Signal transduction histidine-protein kinase BarA
1	ME 1 (isolate 1)	9	166,154	T→G	I	intergenic	Methyl-accepting chemotaxis sensor/transducer protein
1	PE 1 (isolates 1 & 2)	3	108,886	A→G	I	intergenic	HmsT protein/Putative inner membrane protein
2	PE 2 (isolates 1-4)	11	20,324	A→T	NS	GAA→GTA	ATP synthase F0 sector subunit a
2	PE 2 (isolates 1-4)	17	22,967	A→G	NS	GTA→GCA	DNA-directed RNA polymerase beta subunit
3	PE 3 (isolate 1)	3	25,553	A→G	NS	TTC→CTC	PTS system, arbutin, cellobiose, salicin-specific IIB / IIC component
3	PE 3 (isolate 3)	13	101,383	G→C	S	GCG→GCC	Putative secretion permease
1	PE 1 (isolate 3)	3	137,936	T→G	NS	GAC→GCC	Inner membrane protein YfiN
2	PE 2 (isolates 1-4)	3	138,261	T→G	NS	ATC→CTC	Inner membrane protein YfiN
3	PE 3 (isolate 1)	3	138,261	T→G	NS	ATC→CTC	Inner membrane protein YfiN
2	PE 2 (isolates 1-4)	3	270,485	Δ	NS	ΔCGATGC	Signal transduction histidine-protein kinase BarA
3	PE 3 (isolate 1)	3	270,485	Δ	NS	ΔCGATGC	Signal transduction histidine-protein kinase BarA
2	PE 2 (isolate 3)	7	138,605	G→A	S	CAG→CAA	D-galactonate transporter
3	PE 3 (isolate 2 & 3)	9	207,282	G→T	I	intergenic	hypothetical protein/hypothetical protein

Table 1. Intergenic (I), non-synonymous (NS), and synonymous (S) mutations found in the kz19 media-evolved (ME) and predator-evolved (PE) genomes.

There were three mutations in the media-evolved (ME) kz19-1 isolate, including two non-synonymous mutations and one intergenic SNP mutation. One of the non-synonymous mutations was a deletion of 66 base pairs in a gene annotated as the DNA-binding capsular synthesis response regulator RcsB. Additionally, a non-synonymous SNP resulting in a premature stop codon occurred in the signal transduction histidine-protein kinase BarA. An intergenic mutation was identified upstream of the methyl-accepting chemotaxis sensor/transducer protein.

We observed a total of 12 mutations in the predator-evolved (PE) kz19 isolates from our three evolved lines. Of these, the majority (8) were non-synonymous mutations. A six base pair out-of-frame deletion occurred in the signal transduction histidine-protein

kinase BarA gene in all PE isolates from line 2 as well as in the line 3 isolate 3-1. Non-synonymous mutations located in the genomes of one PE isolate from line 1 (1-3), all PE isolates from line 2, and one PE isolate from line 3 (3-1) affected inner membrane protein YfiN. These SNPs occurred in the same position in the genomes of all PE isolates from line 2 and in the line 3 isolate PE 3-1. In the line 1 isolate PE 1-3, the mutation occurred in the same gene but was located in a different position (325 bp away from the mutation found in the other genomes). A non-synonymous SNP in the genomes of all line 2 isolates occurred in the ATP synthase F_0 sector subunit *a*. Another non-synonymous mutation was found in the line 2 genomes, affecting DNA-directed RNA polymerase beta subunit. A non-synonymous SNP in the line 3 isolate PE 3-1 impacted the arbutin-, cellobiose-, and salicin-specific IIB/IIC component of the phosphotransferase system (PTS).

A synonymous mutation found in the line 2 isolate PE 2-3 affected the gene coding for the D-galactonate transporter (DgoT). Another synonymous mutation was found in the line 3 isolate PE 3-3 in a putative secretion permease gene. An intergenic SNP was discovered in the genomes of two of the three PE isolates from line 1 (1-1 and 1-2) upstream of the HmsT protein. Lastly, an intergenic SNP was identified upstream of a hypothetical protein (function unknown) in two of the line 3 isolates (3-2 and 3-3).

3.3 Mutations in within-host-evolved strains

Within-host evolution resulted in six to seven mutations in each of the 10 evolved isolates from our five evolved lines. Remarkably, we identified the same exact six mutations in four out of the five host-evolved lines, i.e., all isolates from lines 1, 2, 4, and

5 presented the same mutations in the same genes (Table 2). In four out of five lines, a non-synonymous mutation occurred in the methyl-accepting chemotaxis protein IV (Table 2). Another non-synonymous mutation altered the respiratory nitrate reductase alpha chain protein. Synonymous mutations occurred in the genes coding for nitrate/nitrite response regulator protein NarL, succinate-semialdehyde dehydrogenase [NAD(P)+], alpha-D-ribose 1-methylphosphonate 5-phosphate C-P lyase and the efflux ABC transporter, permease/ATP-binding protein MdlB.

Contig	Position	Mutation	Type	Description	Annotation
1	655,358	T→A	NS	GAT→GTT	Methyl-accepting chemotaxis protein IV
1	559,265	C→A	NS	GCC→TCC	Respiratory nitrate reductase alpha chain
1	580,034	G→A	S	ATG→ATA	Nitrate/nitrite response regulator protein NarL
7	157,126	C→T	S	CTG→CTA	Succinate-semialdehyde dehydrogenase [NAD(P)+]
12	170,751	C→T	S	CTG→CTA	Alpha-D-ribose 1-methylphosphonate 5-phosphate C-P lyase
15	262,920	C→T	S	GCG→GCA	Efflux ABC transporter, permease/ATP-binding protein MdlB

Table 2. Non-synonymous (NS) and synonymous (S) mutations found in the kz19 host-evolved (HE) line 1, 2, 4, and 5 genomes.

One of our evolved lines, for which we only had one isolate (HE 3-1), presented seven mutations that did not occur in any of the other HE genomes (Table 3). A non-synonymous mutation resulted in an insertion in the gene encoding nitrate/nitrite transporter NarK/NarU. Non-synonymous SNPs were identified in the genes coding for nitrate/nitrite sensor protein NarX, anaerobic glycerol-3-phosphate dehydrogenase subunit A, and in a hypothetical protein. A synonymous SNP also occurred in the same hypothetical protein in which we observed the non-synonomous SNP. Intergenic SNPs occurred on the genes that encode for a putative DNA-binding protein/permease of the

drug/metabolite transporter (DMT) superfamily and the putative protein *Gifsy-2* prophage protein STM1020/STM2620.

Contig	Position	Mutation	Type	Description	Annotation
1	576,678	+T	NS	insertion	Nitrate/nitrite transporter NarK/U
1	578,388	C→T	NS	TCG→TTG	Nitrate/nitrite sensor protein NarX
8	77,368	C→T	NS	GCG→GTG	Anaerobic glycerol-3-phosphate dehydrogenase subunit A
1	165,207	G→T	I	intergenic	Putative DNA-binding protein/Permease of the drug/metabolite transporter (DMT) superfamily
14	182,048	G→A	NS	AGC→AAC	hypothetical protein
14	182,055	G→A	S	AAG→AAA	hypothetical protein
14	182,074	G→A	I	intergenic	hypothetical protein/Gifsy-2 prophage protein STM1020/STM2620

Table 3. Non-synonymous (NS), intergenic (I), and synonymous (S) mutations found in the host-evolved HE 3-1 genome.

CHAPTER IV

DISCUSSION

4.1 Virulence of experimentally-evolved strains

Our results suggest that the presence of a predator increases virulence and the absence of a predator decreases virulence of *S. marcescens* during host infection. These findings support the CEH. Our results contradict the two previous studies that evaluated how predation by *T. thermophila* impacts the virulence of *S. marcescens*, which reported that predation attenuated virulence (Friman et al., 2009; Zhang et al., 2014). However, we believe that the discrepancy between our study and these two previous studies that used the same system (*T. thermophila* and *S. marcescens*) can be attributed to experimental flaws. Both studies used very small sample sizes for their virulence assays and also did not perform replicate survival assays. Zheng et al. (2014) only tested 10 flies in a single vial for each evolved strain and for the ancestral strain, while Friman et al. (2009) did a single survival assay testing approximately 50 moth larvae for each of the treatment groups (evolved, control, and ancestral strains)—again with no replicate assays. Both studies were done in insect host model systems (fruit flies or moths), and from our experience working with insects (both fruit flies and honey bees), we know that in-lab survival can be extremely variable. Thus, multiple replicate assays need to be performed.

For example, if only one replicate virulence assay was performed for each strain and if one host population (i.e., a particular treatment or control group) displayed more

death, then this would be attributed to increased virulence of the tested strain even if it was a result of other factors (diet, temperature, humidity, viral or other pathogen infection, or other factors). Conversely, our results are consistent with the findings of Adiba et al. (2010), which supported the CEH by demonstrating that pathogenic strains of *E. coli* as well as those subject to predation from amoebae in the natural environment resisted predation better than commensal strains. However, Adiba et al. (2010) did not investigate virulence in a host following evolution in the presence of a predator, and we did not test if our strains are more resistant to predation so we cannot directly compare our results to this study.

Here we found that within-host evolution either does not affect or in some cases increases virulence. These results contradict the findings of Mikonranta et al. (2015), which showed that within-host evolution resulted in attenuated virulence. There are a few reasons why we believe that host evolution did not result in attenuated virulence as we predicted. First, we started our within-host evolution experiment using the media-evolved strain, which was predicted have attenuated virulence. Second, our virulence assays compared the host- and media-evolved isolates to each other, so if the media-evolved strain lost virulence and the host-evolved did as well we would not see a different in virulence when comparing them. Third, since KZ19 is an opportunistic pathogen of honey bees, frequently being found at low abundance in the honey bee gut (Raymann et al. 2018), it is already adapted to live in the bee and is not expected to undergo strong selective pressure within the honey bee gut, especially in germ-free bees where there is an absence of competition with other microbes. Also, previous studies of

KZ19 infection in honey bees demonstrated that this strain does not upregulate the immune response and even possibly suppresses the expression of some immune genes (Raymann et al. 2018), suggesting it has already adapted to evade the honey bee immune response.

One main caveat of our study is that our survival assays only compared the predator-evolved isolates to the media-evolved isolate and not the ancestral strain, thus it is undetermined whether the predator-evolved isolates have become more virulent, or if media-evolved virulence is attenuated, or both. Further studies need to be performed by evolving the ancestral strain (rather than the media-evolved strain) in order to accurately determine how within-host evolution impacts virulence.

4.2 Mutations in experimentally-evolved strains

We identified a total of 28 mutations in our experimentally evolved isolates, several of which were in genes that have been associated with virulence in *S. marcescens* or other bacteria. Of these, the majority (16) were non-synonymous mutations, meaning they changed the amino acid sequence of the protein. Synonymous (seven total) and intergenic (five total) mutations were also discovered. Though generally thought to be "silent" and have no effect on gene function, recent studies have revealed that both synonymous and intergenic mutations can have an impact on gene expression and fitness (Bailey et al., 2014; Khademi et al., 2019). Mutations in intergenic regions of DNA were also recently suggested to influence gene expression and contribute to within-host adaptation of bacterial pathogens (Khademi et al., 2019). Specifically, it has been shown that intergenic mutations that occur upstream of transcriptional start sites (in regulatory

elements) can affect virulence, including antibiotic resistance and iron acquisition (Khademi et al., 2019; Marvig et al., 2014). Therefore, all the mutations we identified in our evolved strains could have an impact on adaptation and fitness, including virulence. However, here, we will only discuss the non-synonomous mutations (as they definitely impact function and expression of proteins) that have been associated with virulence. Details on the functions (if known) of the genes in which we found synonymous mutations or flanking intergenic mutations are described in Appendix B. Because no previous experimental evolution studies testing the CEH have evaluated the mutations that occurred in the evolved lines, we are unable to compare our findings to other studies.

4.2.1 Media-evolved mutations

Of the three mutations identified in ME isolate (1-1) two were non-synonymous and have been implicated in virulence. The non-synonymous mutation annotated as the DNA-binding capsular synthesis response regulator RcsB was a 66 base pair deletion, indicating that this gene lost function. The premature stop codon occurring in the signal transduction histidine-protein kinase BarA also resulted in loss of gene function. RcsB is a global response regulator and is the final step in the RcsCDB phosphorelay system, which is activated by cell envelope stress, and is conserved in *Enterobacteriaceae* such as *S. marcescens* (Casino et al., 2018; Filippova et al., 2018). RcsB has been shown to play an important role in virulence, including in the synthesis of biofilms, capsules, and flagella and in antibiotic resistance (Filippova et al., 2018). The BarA gene activates the response regulator RcsB and would no longer be needed if RcsB is not functional. Taken together, the loss of function of RcsB and BarA indicate that the RcsCDB phosphorelay

system is no longer active in the media-evolved strain. The loss of these genes and this pathway could be due to the lack of selective pressure to maintain costly defense/virulence mechanisms in a predator-free environment and is likely the reason for attenuated virulence in the media-evolved strain. However, these mutations will need to be experimentally verified to confirm their role in virulence.

4.2.2 Predator-evolved mutations

Of the 12 mutations we identified in our predator evolved lines eight were non-synonymous, two of which occurred in genes previously associated with virulence. A six base pair out-of-frame deletion occurred in the signal transduction histidine-protein kinase BarA gene in all isolates from line 2 as well as in the line 3 isolate 3-1. Isolates from the same evolved line (e.g. 1, 2, or 3) are likely clones rather than different strains. Thus, isolates from the same evolved line that have the same mutations in the exact same positions do not likely indicate parallel evolution. However, isolate PE 3-1 has an identical non-synonymous mutation as all the isolates from line 2, affecting BarA, indicating that parallel evolution has occurred. As described above, BarA activates the response regulator RcsB, which plays an important role in the synthesis of biofilms, capsules, and flagella. However, there are multiple proteins that can activate RcsB, aside from BarA, and the use of each depends on environmental conditions (Salvail & Groisman, 2020). Therefore, the PE isolates have lost the ability to affect RcsB expression via BarA, potentially because using another RcsB regulator is more efficient and/or important in the presence of a predator. The fact that this exact deletion was

observed in two of our lines, indicates that BarA is not important for adaptation to the presence of a predator.

The non-synonymous SNPs located in the genomes of one isolate from line 1 (1-3), all isolates from line 2, and one isolate from line 3 (3-1) affected inner membrane protein YfiN and occurred in the same position in the genomes of all isolates from line 2 and in the line 3 isolate PE 3-1 (parallel evolution). In the line 1 isolate PE 1-3, the mutation occurred in the same gene but was located in a different position (i.e., this does not denote parallel evolution). Previous studies have elucidated YfiN's importance in biofilm formation, reduction in swimming motility, and cell division arrest of bacteria (Giardina et al., 2013; Kim & Harshey, 2016; Sanchez-Torres et al., 2011). Multiple discoveries of non-synonymous mutations in this gene in different PE lines (some being cases of parallel evolution) suggest that YfiN plays an important role in adaptation to predators, and thus, virulence.

Other non-synonymous mutations were identified in genes that have not been implicated in virulence such as ATP synthase F_0 sector subunit *a*; DNA-directed RNA polymerase beta subunit; and PTS system, arbutin-, cellobiose-, salicin-specific IIB / IIC component. It is not clear how these mutations could impact resistance to predation but they could have an impact on fitness. All mutations identified will need to be experimentally tested to determine their role in virulence.

4.2.3 Within-host-evolved mutations

There were six to seven mutations in each of the 10 within-host-evolved isolates from our five evolved lines, with four out of the five lines harboring six identical

mutations (i.e., all isolates from lines 1, 2, 4, and 5 presented the same mutations in the same genes). This indicates that parallel evolution has occurred four times during within-host evolution (the isolates from the same line are likely clones and do not represent real cases of parallel evolution). Of the six mutations identified across the four evolved lines, only two were non-synonymous and occurred in genes that have been implicated in chemotaxis and to adaptation to anaerobic environments.

In four out of five lines, a non-synonymous mutation occurred in the methyl-accepting chemotaxis protein (MCP) IV. Bacteria use chemotaxis to respond to and move toward chemicals in the environment. MCPs are involved in the regulation of several virulence factors including the synthesis of biofilms, flagella, and toxins in many pathogens (Salah Ud-din et al., 2017). MCP IV has been shown to be specifically involved in sensing dipeptides (Liu & Parales, 2008). Thus, it is possible that the ability to sense and move towards dipeptides is important within the bee gut environment.

In the four parallel lines, another non-synonymous mutation altered the respiratory nitrate reductase alpha chain protein. When oxygen is lacking in the environment, some bacteria can employ nitrate as an electron acceptor using the nitrate reductase enzyme complex. The respiratory nitrate reductase alpha chain is the actual site of nitrate reduction and NarL, the nitrate/nitrite response regulator protein, controls transcription of nitrate reductase and formate dehydrogenase-N when nitrate is present, i.e., under anaerobic conditions (Shivakumar et al., 2014). It is worth noting that we also observed a synonymous mutation in NarL in these four parallel lines. It is not clear how these mutations would impact fitness within the host environment, as nitrate is not

expected to be present in the honey bee gut environment, especially in lab reared "germ-free" bees. However, it does make sense that there would be selective pressure on genes involved in survival in anaerobic conditions since the bee gut environment is low in oxygen (Zheng et al., 2017). It is possible that the function of these genes in *S. marcescens* may be different from that of other organisms or that they have multiple functions in anaerobic environments that have not been recognized.

Isolate HE 3-1 presented seven mutations that did not occur in any of the other HE genomes. Some of these occurred in genes in the same Nar (respiratory nitrate reductase) pathway in which we found mutations in the other four HE lines, suggesting this pathway may be important for adaptation within a host. An insertion, which would cause a frameshift in NarK/NarU, occurred in HE isolate 3-1 suggesting the gene is no longer functional. NarK and NarU are nitrate/nitrite transporters (Yan et al., 2013). While NarK is more abundant in a nitrate-rich environment, during nutrient starvation NarU is more prevalent (Clegg et al., 2006). It is unclear if this gene is NarK or NarU based on the annotation, or if there are multiple homologs of this protein within the genome. This needs to be further investigated. A non-synonymous SNP also occurred in this isolate in the gene coding for NarX. NarX signals environmental nitrate or nitrite availability to response regulator proteins NarL and NarP, which, in turn, triggers anaerobic respiration (Williams & Stewart, 1997). Thus, the non-synonymous SNP in NarX could be related to the insertion identified in NarK/NarU. Moreover, the fact that mutations in genes involved in the Nar pathway were also observed in the other lines suggests that this pathway is being inactivated or upregulated in the host-evolved strains.

However, it is not clear if the Nar pathway is critical or nonessential for adaptation to the host environment.

The fact that four out of five host-evolved lines carry the same mutations in the same locations could indicate these genes are important for adaptation to the host environment and indicate parallel evolution has occurred. However, six instances of parallel evolution, with four of them being synonymous mutations, warrants caution. One alternative theory is that these are not mutations but instead instances of homologous recombination. Although our honey bees are considered "germ-free", they typically still have low levels of commensal environmental bacteria present in their guts. Environmental bacteria frequently found in honey bee guts are *Klebsiella* and *Enterobacteria* (Raymann & Moran 2018), both of which are close relatives to *Serratia*. Therefore, it is possible that our KZ19 strains recombined with one of these, or another, bacteria present in our "germ-free" bees, resulting in the observed nucleotide changes. This hypothesis warrants further testing.

4.3 Conclusion

We experimentally evolved a multidrug-resistant, opportunistic pathogen of plants and animals (*S. marcescens*) to test the CEH and determine how adaptation to different environments impact virulence. We found that evolution in the presence of a eukaryotic predator increases virulence in *S. marcescens* compared to a media-evolved isolate—results that agree with the coincidental evolution hypothesis. Our hypothesis that within-host evolution results in attenuated virulence still needs further testing. For example, the KZ19 ancestral strain, rather than the media-evolved strain, should be used

to start the within-host evolution experiments. Also, more media-evolve strains need to be sequenced, and additional virulence assays comparing the host-, predator- and media-evolved strains to the ancestral strain need to be performed. Future studies should also involve predator assays—facing our predator-evolved lines against *T. thermophila* as well fitness assays. This will help determine if the evolved lines are now better able to withstand predation compared to the ancestor and will test fitness in terms of growth rate. Furthermore, the mutations we found need to be experimentally validated to determine if they play a role in virulence by genetically engineering strains with the identified mutations and testing for virulence in honey bees.

In summary, our findings support the CEH and show that experimental evolution in the presence or absence of a predator results in mutations in the genes involved in virulence in *S. marcescens*. This research identified mutations involved in virulence in an opportunistic pathogen, including biofilm formation, swimming motility, and antibiotic resistance. We also demonstrated that experimental evolution can be successfully performed *in vivo* in the honey bee, proving it is a great model system to study OP evolution as well as OP-host interactions. Overall, the findings presented here may aid in developing targeted drug treatments to combat an increasingly persistent, MDR pathogen that infects plants and animals, including humans.

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APPENDIX A.

SUPPLEMENTAL FIGURES

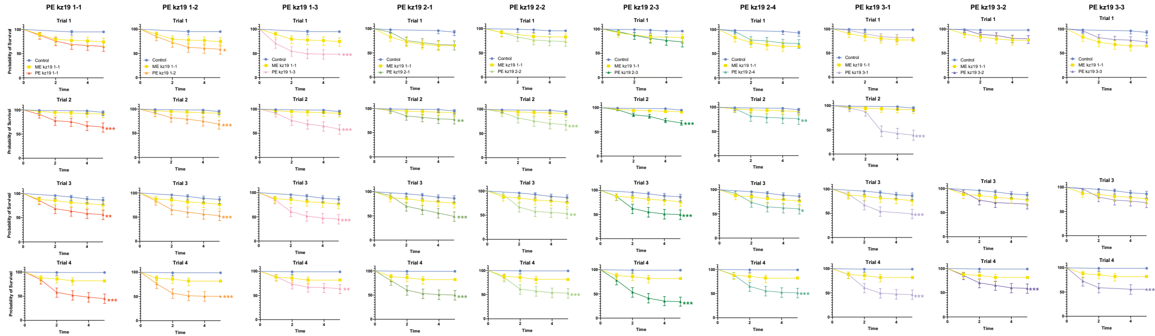

Figure A1. Probability of survival (y-axis) of honey bees for Objective 1 (all trials). Bees fed KZ19 predator-evolved (PE) isolates showed an increase in mortality over five days compared to the control (blue line) or the media-evolved (ME) KZ19 isolate (yellow line).

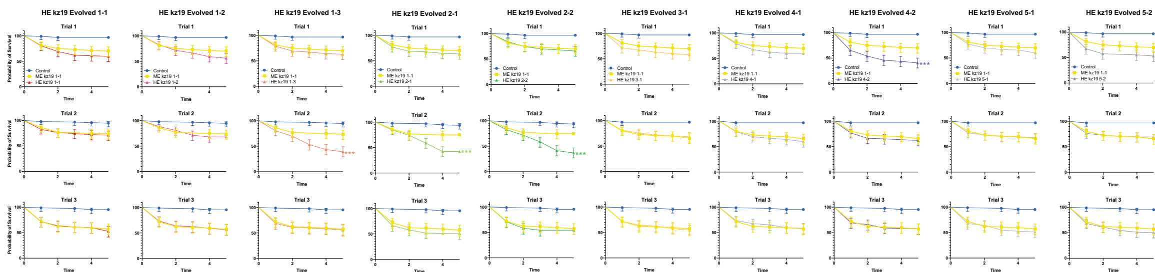

Figure A2. Probability of survival (y-axis) of honey bees for Objective 3. Bees fed KZ19 host-evolved (HE) isolates showed an increase in mortality over five days compared to the control (blue line) or the media-evolved (ME) KZ19 isolate (yellow line).

Strain	Contig	Position	Ref	New	Frequency	Reads	Product
ME_kz19-1	13	457503	66bp	Δ66bp	100%	63	DNA-binding capsular synthesis response regulator RcsB
ME_kz19-1	3	269,652	G	A	98.8%	80	Signal transduction histidine-protein kinase BarA
ME_kz19-1	9	166,154	T	G	100%	48	Methyl-accepting chemotaxis sensor/transducer protein
PE_kz19-1-1	3	108,890	A	G	100%	46	HmsT protein/Putative inner membrane protein
PE_kz19-1-2	3	108,890	A	G	100%	161	HmsT protein/Putative inner membrane protein
PE_kz19-1-3	3	137,936	T	G	100%	26	Inner membrane protein YfiN
PE_kz19-2-1	11	20,324	A	T	100%	54	ATP synthase F0 sector subunit a
PE_kz19-2-1	17	22,967	A	G	100%	68	DNA-directed RNA polymerase beta subunit
PE_kz19-2-1	3	138,261	T	G	100%	60	Inner membrane protein YfiN
PE_kz19-2-1	3	270,484	6bp	Δ6bp	100%	57	Signal transduction histidine-protein kinase BarA
PE_kz19-2-1	11	20,324	A	T	100%	66	ATP synthase F0 sector subunit a
PE_kz19-2-2	17	22,967	A	G	100%	75	DNA-directed RNA polymerase beta subunit
PE_kz19-2-2	3	138,261	T	G	96.9%	32	Inner membrane protein YfiN
PE_kz19-2-3	3	270,484	6bp	Δ6bp	100%	60	Signal transduction histidine-protein kinase BarA
PE_kz19-2-3	11	20,324	A	T	100%	88	ATP synthase F0 sector subunit a
PE_kz19-2-3	17	22,967	A	G	100%	81	DNA-directed RNA polymerase beta subunit
PE_kz19-2-3	3	138,261	T	G	100%	52	Inner membrane protein YfiN
PE_kz19-2-3	3	270,484	6bp	Δ6bp	100%	45	Signal transduction histidine-protein kinase BarA
PE_kz19-2-4	11	20,324	A	T	100%	34	ATP synthase F0 sector subunit a
PE_kz19-2-4	17	22,967	A	G	100%	27	DNA-directed RNA polymerase beta subunit
PE_kz19-2-4	3	138,261	T	G	100%	24	Inner membrane protein YfiN
PE_kz19-2-4	3	270,484	6bp	Δ6bp	100%	35	Signal transduction histidine-protein kinase BarA
PE_kz19-3-1	3	25,553	A	G	93.2%	59	PTS system, arbutin-, cellobiose-, and salicin-specific IIB/IIC component
PE_kz19-3-1	3	138,261	T	G	100%	49	Inner membrane protein YfiN
PE_kz19-3-1	3	270,484	6bp	Δ6bp	100%	63	Signal transduction histidine-protein kinase BarA
PE_kz19-3-2	9	207,282	G	T	100%	61	hypothetical protein
PE_kz19-3-3	9	207,282	G	T	100%	24	hypothetical protein

Table A1. Mutations found in the predator- and media-evolved strains via BreSeq.

Strain	Contig	Position	Ref	New	Frequency	Reads	Product
HE_kz19 1-1	1	559,265	C	A	100%	22	Respiratory nitrate reductase alpha chain
HE_kz19 1-1	1	580,034	G	A	96.4%	28	Nitrate/nitrite response regulator protein NarL
HE_kz19 1-1	1	655,358	T	A	100%	33	Methyl-accepting chemotaxis protein IV
HE_kz19 1-1	12	170,751	C	T	100%	58	Alpha-D-ribose 1-methylphosphonate 5-phosphate C-P lyase
HE_kz19 1-1	15	262,920	C	T	100%	53	Efflux ABC transporter, permease/ATP-binding protein MdlB
HE_kz19 1-1	7	157,126	C	T	100%	28	Succinate-semialdehyde dehydrogenase [NAD(P)+]
HE_kz19 1-2	1	559,265	C	A	100%	6	Respiratory nitrate reductase alpha chain
HE_kz19 1-2	1	580,034	G	A	100%	5	Nitrate/nitrite response regulator protein NarL
HE_kz19 1-2	1	655,358	T	A	100%	6	Methyl-accepting chemotaxis protein IV
HE_kz19 1-2	12	170,751	C	T	100%	5	Alpha-D-ribose 1-methylphosphonate 5-phosphate C-P lyase
HE_kz19 1-3	1	559,265	C	A	100%	35	Respiratory nitrate reductase alpha chain
HE_kz19 1-3	1	580,034	G	A	100%	30	Nitrate/nitrite response regulator protein NarL
HE_kz19 1-3	1	655,358	T	A	100%	38	Methyl-accepting chemotaxis protein IV
HE_kz19 1-3	12	170,751	C	T	100%	52	Alpha-D-ribose 1-methylphosphonate 5-phosphate C-P lyase
HE_kz19 1-3	15	262,920	C	T	100%	31	Efflux ABC transporter, permease/ATP-binding protein MdlB
HE_kz19 1-3	7	157,126	C	T	100%	41	Succinate-semialdehyde dehydrogenase [NAD(P)+]
HE_kz19 2-1	1	559,265	C	A	100%	67	Respiratory nitrate reductase alpha chain
HE_kz19 2-1	1	580,034	G	A	98.3%	60	Nitrate/nitrite response regulator protein NarL
HE_kz19 2-1	1	655,358	T	A	100%	50	Methyl-accepting chemotaxis protein IV
HE_kz19 2-1	12	170,751	C	T	100%	73	Alpha-D-ribose 1-methylphosphonate 5-phosphate C-P lyase
HE_kz19 2-1	15	262,920	C	T	100%	60	Efflux ABC transporter, permease/ATP-binding protein MdlB
HE_kz19 2-1	7	157,126	C	T	100%	53	Succinate-semialdehyde dehydrogenase [NAD(P)+]
HE_kz19 2-2	1	559,265	C	A	100%	55	Respiratory nitrate reductase alpha chain
HE_kz19 2-2	1	580,034	G	A	100%	52	Nitrate/nitrite response regulator protein NarL
HE_kz19 2-2	1	655,358	T	A	100%	49	Methyl-accepting chemotaxis protein IV
HE_kz19 2-2	12	170,751	C	T	100%	91	Alpha-D-ribose 1-methylphosphonate 5-phosphate C-P lyase
HE_kz19 2-2	15	262,920	C	T	100%	74	Efflux ABC transporter, permease/ATP-binding protein MdlB
HE_kz19 2-2	7	157,126	C	T	100%	55	Succinate-semialdehyde dehydrogenase [NAD(P)+]
HE_kz19 3-1	1	165,207	G	T	100%	36	Permease of the drug/metabolite transporter (DMT) superfamily
HE_kz19 3-1	1	576,678	-	T	98%	49	Nitrate/nitrite transporter NarK/U
HE_kz19 3-1	1	578,388	C	T	100%	58	Nitrate/nitrite sensor protein NarX
HE_kz19 3-1	14	182,048	G	A	100%	13	hypothetical protein
HE_kz19 3-1	14	182,055	G	A	100%	15	hypothetical protein
HE_kz19 3-1	14	182,074	G	A	100%	23	Gifsy-2 prophage protein STM1020/STM2620
HE_kz19 3-1	8	77,368	C	T	100%	84	Anaerobic glycerol-3-phosphate dehydrogenase subunit A
HE_kz19 4-1	1	559,265	C	A	100%	44	Respiratory nitrate reductase alpha chain
HE_kz19 4-1	1	580,034	G	A	92.3%	26	Nitrate/nitrite response regulator protein NarL
HE_kz19 4-1	1	655,358	T	A	97.6%	41	Methyl-accepting chemotaxis protein IV
HE_kz19 4-1	12	170,751	C	T	100%	70	Alpha-D-ribose 1-methylphosphonate 5-phosphate C-P lyase
HE_kz19 4-1	15	262,920	C	T	97.9%	47	Efflux ABC transporter, permease/ATP-binding protein MdlB
HE_kz19 4-1	7	157,126	C	T	100%	55	Succinate-semialdehyde dehydrogenase [NAD(P)+]
HE_kz19 4-2	1	559,265	C	A	96.4%	28	Respiratory nitrate reductase alpha chain
HE_kz19 4-2	1	580,034	G	A	96.4%	28	Nitrate/nitrite response regulator protein NarL
HE_kz19 4-2	1	655,358	T	A	100%	41	Methyl-accepting chemotaxis protein IV
HE_kz19 4-2	12	170,751	C	T	100%	50	Alpha-D-ribose 1-methylphosphonate 5-phosphate C-P lyase
HE_kz19 4-2	15	262,920	C	T	100%	54	Efflux ABC transporter, permease/ATP-binding protein MdlB
HE_kz19 4-2	7	157,126	C	T	100%	42	Succinate-semialdehyde dehydrogenase [NAD(P)+]
HE_kz19 5-1	1	559,265	C	A	100%	21	Respiratory nitrate reductase alpha chain
HE_kz19 5-1	1	580,034	G	A	100%	25	Nitrate/nitrite response regulator protein NarL
HE_kz19 5-1	1	655,358	T	A	95.5%	22	Methyl-accepting chemotaxis protein IV
HE_kz19 5-1	12	170,751	C	T	100%	35	Alpha-D-ribose 1-methylphosphonate 5-phosphate C-P lyase
HE_kz19 5-1	15	262,920	C	T	100%	34	Efflux ABC transporter, permease/ATP-binding protein MdlB
HE_kz19 5-1	7	157,126	C	T	100%	30	Succinate-semialdehyde dehydrogenase [NAD(P)+]
HE_kz19 5-2	1	559,265	C	A	100%	35	Respiratory nitrate reductase alpha chain
HE_kz19 5-2	1	580,034	G	A	100%	26	Nitrate/nitrite response regulator protein NarL
HE_kz19 5-2	1	655,358	T	A	97.6%	42	Methyl-accepting chemotaxis protein IV
HE_kz19 5-2	12	170,751	C	T	100%	33	Alpha-D-ribose 1-methylphosphonate 5-phosphate C-P lyase
HE_kz19 5-2	15	262,920	C	T	100%	39	Efflux ABC transporter, permease/ATP-binding protein MdlB
HE_kz19 5-2	7	157,126	C	T	100%	32	Succinate-semialdehyde dehydrogenase [NAD(P)+]

Table A2. Mutations found in the host-evolved strains via BreSeq.

APPENDIX B.

DESCRIPTIONS OF THE FUNCTIONS OF GENES MUTATED IN THE EVOLVED LINES

RcsB. RcsB is a global response regulator and is the final step in the RcsCDB phosphorelay system which is activated by cell envelope stress, and is conserved in *Enterobacteriaceae* such as *S. marcescens* (Casino et al., 2018; Filippova et al., 2018). RcsB has been shown to play an important role in virulence, including in the synthesis of biofilms, capsules, and flagella and in antibiotic resistance (Filippova et al., 2018). For example, in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, it inhibits flagellar expression while simultaneously initiating biofilm production (Casino et al., 2018). In *E. coli* and other members of the order *Enterobacterales*, RcsB has been implicated in promoting capsule production while inhibiting motility (Wall et al., 2018). In *S. marcescens*, antimicrobials from competitors trigger its RcsB-dependent Type VI secretion system, causing it to release toxins that eliminate this competition (Lazzaro et al., 2017).

BarA. The BarA is an activator of the global response regulator RcsB. However, RcsB activation requires RcsC and RcsD but not BarA (Salvail & Groisman, 2020). Moreover, there are multiple proteins that can activate RcsB, aside from BarA, and the use of each has been shown to depend on environmental conditions (Salvail & Groisman, 2020).

HmsT. Previous studies have identified HmsT as crucial to flea-to-mammal transmission of *Yersinia pestis*—the causative agent of bubonic, pneumonic, and septicemic plagues

(Jones et al., 1999; Kirillina et al., 2004; Sun et al., 2012; Zhu et al., 2016). Transmission of *Y. pestis* from the Oriental rat flea, *Xenopsylla cheopis*, to mammals requires the haemin storage (Hms) phenotype, characterized by biofilm buildup in the foregut. This biofilm blocks nutrient accession in regions beyond the foregut, and starvation ensues. Fleas bite a host multiple times in a vain attempt at feeding, which, in turn, transmits the pathogen (Jones et al., 1999; Zhu et al., 2016). HmsT is one of two diguanylate cyclase enzymes responsible for biofilm synthesis in Y. pestis (Sun et al., 2012).

Inner membrane protein YfiN. Previous studies have elucidated YfiN's importance in biofilm formation, reduction in swimming motility, and cell division arrest of bacteria (Giardina et al., 2013; Kim & Harshey, 2016; Sanchez-Torres et al., 2011). The diguanylate cyclase YfiN synthesizes second messenger Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) in response to cell stress (Xu et al., 2016) and is conserved in Gram-negative bacteria (Kim & Harshey, 2016) including *Pseudomonas aeruginosa*, *E. coli*, and *S. marcescens*. An increase in c-di-GMP concentration results in reduced swimming motility and an increase in biofilm production in *P. aeruginosa* (Smet et al., n.d.), the causative agent of cystic fibrosis pneumonia. In *E. coli*, c-di-GMP halts cell division during immune system or antibiotic attacks (Kim & Harshey, 2016).

Methyl accepting chemotaxis sensor/transducer protein. Bacteria utilize receptors known as methyl-accepting chemotaxis proteins (MCPs) to sense and respond to environmental stimuli. MCPs are involved in the regulation of virulence factors including the synthesis of biofilms, flagella, and toxins in many pathogens (Salah Ud-din et al., 2017).

ATP synthase F_0 sector subunit *a*. ATP synthases produce adenosine triphosphate (ATP) from adenosine diphosphate and inorganic phosphate during oxidative phosphorylation (Guo et al., 2019). Composed of sectors F_1 and F_0, ATP synthases translocate protons across membranes via an electrochemical gradient. In bacteria, the F_0 sector is a protein located in the cytoplasmic inner membrane, and is made up of several subunits, including subunit *a* (Dimroth, 2000; Hermolin & Fillingame, 1995). I don't understand how subunit a functions from the papers I have read☹ I think it just pumps protons through the membrane??

DNA-directed RNA polymerase β subunit. During transcription, RNA polymerase copies a sequence of DNA in order to produce RNA. The β subunit forms an enzyme complex with the β' subunit, where DNA enters the active site for transcription (Sutherland & Murakami, 2018). The β subunit is also where single stranded RNA exits the complex (Sutherland & Murakami, 2018). Rifampin (RMP)—an antibiotic used to treat tuberculosis—targets the β subunit of *Mycobacterium tuberculosis*, and works by blocking RNA elongation (Sutherland & Murakami, 2018). Antibiotic resistance to salinamide A can be traced to mutations in the genes that code for RNA polymerase (Degen et al., 2014).

PTS system, arbutin-, cellobiose-, and salicin-specific IIB/IIC component. In *E. coli*, the the arbutin-, cellobiose-, and salicin-specific IIB/IIC component of the phosphotransferase system (PTS) is responsible for transporting and phosphorylating glucose for carbon and energy, playing a key role in utilizing glucose instead of other carbon sources (Escalante et al., 2012). In media containing glucose (such as that used in

the predator evolution experiments), enzyme IIC transports sugar to the IIB enzyme, which phosphorylates it (Escalante et al., 2012). I think this paper (Escalante) says carbon catabolite repression (CCR) also plays a role in virulence and biofilm formation, but I may be misunderstanding: CCR may have nothing to do with IIB/IIC components.

D-galactonate transporter. D-galactonate transporter (DgoT) belongs to the solute carrier 17 (SLC17) family of transporters (Leano et al., 2019). While the function of DgoT is not defined in bacteria, SLC17 genes lie within operons involved in sugar metabolism (Leano et al., 2019). DgoT is a symporter, coupling the movement of protons and D-galactonate (Leano et al., 2019).

Methyl-accepting chemotaxis protein IV. Methyl-accepting chemotaxis protein IV is involved in sensing dipeptides, including the pyrimidines thymine and uracil (Liu & Parales, 2008). As described above, bacteria employ receptors which alert them to environmental changes. In response, bacteria change the direction of swimming motility towards attractants or away from repellants such as antibiotics.

Respiratory nitrate reductase alpha chain. When oxygen is lacking in the environment, bacteria can employ nitrate as an electron acceptor. Nitrate reduction occurs at the alpha chain (Blasco et al., n.d.).

Nitrate/nitrite response regulator protein NarL. The nitrate/nitrite response regulator protein Nar controls transcription of nitrate reductase and formate dehydrogenase-N when nitrate is present (i.e., under anaerobic conditions), such as occurs during *Mycobacterium tuberculosis* infection (Shivakumar et al., 2014).

Succinate-semialdehyde dehydrogenase [NAD(P)+]. Succinate-semialdehyde dehydrogenase [NAD(P)+] (SSDH) is responsible for oxidizing succinate-semialdehyde to succinate (Fuhrer et al., 2007) and for its ability to use various growth substrates (Zheng et al., 2013). SSDH plays an important role in metabolism and survival under low nutrient conditions (Fuhrer et al., 2007), and acid tolerance in bacteria (Feehily & Karatzas, 2013). Since the gut environment is highly acidic, acid tolerance or resistance is important for both pathogens and commensal bacteria. However, increased acid and stress resistance have also been shown to impair colonization or virulence (Feehily & Karatzas, 2013). A mutation in SSDH could impact the ability of the host-evolved isolates to tolerate low pH.

Alpha-D-ribose 1-methylphosphonate 5-phosphate C-P lyase. Alpha-D-ribose 1-methylphosphonate 5-phosphate C-P lyase is an enzyme that degrades phosphonate (Wang et al., 2017).

Efflux ABC transporter, permease/ATP-binding protein MdlB. Efflux ABC transporters pump substances including xenobiotics out of cells. MdlB is one such transporter that enables bacteria to pump isopentenol out of the cell (Jensen et al., 2017).

Nitrate/nitrite transporter NarK/NarU. The ability to convert atmospheric nitrogen to ammonium during anaerobic conditions is vital to bacteria that use it in the production of amino acids. Nitrogen is available to bacteria in the forms of nitrate and nitrite, which are reduced to ammonium (Yan et al., 2013). NarK and NarU are nitrate/nitrite transporters (Yan et al., 2013), belonging to the ATP-binding cassette (ABC) transporter family (Fukuda et al., 2015). While NarK is more abundant in a nitrate-rich environment,

during nutrient starvation NarU is more prevalent (Clegg et al., 2006). In enteric bacteria such as E. coli, the narU operon is highly conserved—suggesting that NarU accumulation presents a selective advantage to bacteria that survive in anaerobic conditions (Clegg et al., 2006).

Nitrate/nitrite sensor protein NarX. The nitrate/nitrite sensor protein NarX is part of a two-component regulatory system that signals environmental nitrate or nitrite availability to response regulator proteins NarL and NarP, which, in turn, triggers anaerobic respiration (Williams & Stewart, 1997).

Anaerobic glycerol-3-phosphate dehydrogenase subunit A. Glycerol can be used by bacteria for carbon and energy, and its metabolism by pathogens such as *Mycoplasma pneumoniae* produces toxic hydrogen peroxide (Blötz & Stülke, 2017). An intermediate of glycerol metabolism is glycerol-3-phosphate, which is anaerobically oxidized by glycerol-3-phosphate dehydrogenase subunit A (GlpA) to dihydroxyacetone (Koga et al., 2019).

DNA-binding protein/permease of the drug/metabolite transporter (DMT) superfamily. Proteins belonging to the DMT superfamily act as efflux pumps, contributing to antibiotic resistance (Jack et al., 2001).

***Gifsy-2* prophage protein STM1020/STM2620.** *Gifsy-2* is a prophage identified in *Salmonella enterica* serovar Typhimurium that contributes to infection in mice (Figueroa-Bossi & Bossi, 1999). *Gifsy-2* carries sodC, a gene that encodes for a superoxide dismutase linked to defense against macrophages in bacteria (Figueroa-Bossi & Bossi, 1999).