EXPERIMENTAL EVOLUTION OF A BACTERIAL SYMBIONT TO ITS HOST'S ENVIRONMENT

by

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Karen Guillemin

The bacteria that live in our guts, and those of other vertebrates, affect our health in a myriad of ways, from aiding in digestion to training our immune system. However, how bacteria first colonize the gut is little-understood. In particular, environment seems to play an important role in host colonization, especially in aquatic organisms. I proposed investigating environmental adaptation to find novel mechanisms for host colonization. I hypothesized that adaptation of a bacterial symbiont to its host's environment increases host colonization. I tested this hypothesis via experimental evolution by serially passaging a strain of *Aeromonas veronii,* a zebrafish gut isolate, in fish-conditioned water to quickly and non-specifically find new genes that could affect host colonization. Surprisingly, I found that while the evolved strains grew to higher population densities in the water than the ancestor, these strains had variable gut colonization fitness. In fact, one strain had significantly reduced gut colonization fitness. Genome sequencing revealed that this strain had mutations affecting motility and Type I secretion system membrane protein genes. I recreated the latter mutation in the wildtype bacterial strain and found that it increased *Aeromonas* fitness in fish water, however gut colonization was comparable to the wildtype. This suggests that other

mutations in the evolved isolate, presumably those in the motility genes, are responsible for the reduced host colonization. Future work will further investigate motility mutations among others. This work contributes to our understanding of host colonization dynamics and can lead to the development of probiotics to improve human health.

Acknowledgements

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Introduction

All animals support an ecosystem of microbes which compose their microbiome. Recent advances in sequencing technologies have allowed researchers to investigate these diverse communities of microbes that colonize the skin and digestive tract. As animals evolved, this plethora of bacterial species evolved with them, developing specialized niches that are shaped by interspecies competition as well as the host's immune system. These forces led to intimate relationships between symbionts and the host, many of which are mutually beneficial. For example, the microbes can help protect the host from enteric disease while the host provides the bacterial species with nutrients and a stable environment. Increasingly, enteric bacteria have been shown to play important roles in human digestion, nutrition, development, and even behavior (McFall-Ngai et al., 2013).

The strategies that bacteria use to facilitate colonization of the host is an important and little-understood area of investigation in the field of host-microbe interactions, yet it has wide implications for host health. What strategies lead one strain to colonize better than another? How is this encoded in the bacterial genome? The answers to these questions could lead to the development of better probiotics that could treat a variety of diseases ranging from inflammatory bowel diseases (Frank et al., 2007) to obesity (Turnbaugh et al., 2009). Animal models can help to answer these questions, which could then lead to advances in human health.

The Guillemin lab uses zebrafish (*Danio rerio*) as a model to study host-microbe interactions. One focus is on understanding the interactions between the host's immune system and the gut microbiota. Researchers in the lab have found that one natural gut

isolate, *Aeromonas veronii* ZOR0001 hereafter referred to as *Aer01,* alone was able to induce the complete immune response seen in zebrafish raised with an otherwise complete microbiome (Rolig et al., 2015). Ongoing experiments have focused on using this biologically relevant bacterial strain to study the bacterial traits important for colonization. They use experimental evolution, where evolution of a bacterial population is used to find which genes are important for living in a particular environment, in this case the zebrafish gut, which is an efficient, and targeted way to find a wide variety of mutations. These experiments involved passaging of *Aeromonas* in zebrafish to generate evolved isolates with increased ability to colonize the gut. In this passaging, larval fish that are raised germ-free (in the absence of microbes), are inoculated with gut-associated *Aeromonas* populations for several days, then the guts are dissected out and the population of *Aeromonas* is transferred to the next group of larval zebrafish, in a process called serial passaging. Interestingly, the evolved isolates, which displayed increased within-host colonization capacities, were also observed to reach higher population densities in the water that the fish were living in, which from this point on will be referred to as fish-conditioned embryo media (FC-EM). This suggests that environmental adaptation is also playing a role in increasing host colonization, but there are other confounding mutations in these particular strains to find out what exactly in the host environment is leading to this change.

I wanted to focus directly on how environmental adaptation affects a bacterium's host-colonization ability. Does a symbiotic strain only need to grow to higher densities, and thus increase its host's sampling rate, in order to colonize the gut more effectively? I hypothesized that evolved populations that grow to higher densities

in this environment have more opportunities to colonize the gut, perhaps by being sampled more often by the host, and thus would reach higher densities in the gut than the ancestral strain. The goal of this project is to identify bacterial traits, and ultimately genes, that are important for host colonization.

To this end, I have used experimental evolutionary techniques to serially passage a strain of *Aeromonas veronii* in FC-EM. I found that these strains adapted to the low-nutrient conditions of FC-EM and grew to higher densities in this environment compared to the ancestral strain. To our surprise, I found that while the evolved strains grew to higher population densities in the water compared to the ancestor, they had variable changes in host gut colonization. Of the three isolates surveyed, one colonized the gut significantly better, one colonized the gut significantly worse, and one had no change compared to the ancestor. These changes in the physical traits (phenotype) of the strains indicate they have mutations in genes critical for gut colonization. The most common predicted mutation was in the isolate that had a significant decrease in gut colonization. This mutation was in resulted in the loss of a loss of Type I secretion system membrane protein (*prsE*) and seems to contribute to these evolved isolates' ability to reach higher densities in FC-EM, but does not change host colonization ability. Thus, it is likely that predicted rearrangements in these genomes, especially in genes that affect motility might be causing this significant decrease in host colonization. Future work will include further genetic manipulation, perhaps in genes affecting motility to investigate the function of the other mutations found through experimental evolution passaging.

Background

The Human Microbiome

The mature human gut microbiome is primarily composed of bacteria, but also includes members of the other two domains of life, Archaea and Eukarya, along with viruses (Subramanian et al., 2015). Until a few decades ago, the only way to measure the composition of the gut microbiome was through culture-dependent methods. This required the ability of researchers in the lab to replicate the exact conditions required for the growth of these strains, which is very challenging and so the vast majority of bacterial strains remained undetected. Advances in DNA sequencing technology have enabled culture-independent methods to examine microbiomes. Sequencing one specific region of the ribosome (16s rRNA), which acts as a barcode for individual species of bacteria, allows researchers to identify the composition of the gut microbiota through metagenomics analysis. The mature gut is thought to harbor up to 500 species of bacteria, which diet and other environmental factors can influence, and these factors have been the focus of much research (Stecher & Hardt, 2011).

In addition to contributing to host metabolic processes, the mature gut microbiota has also been shown to play a significant role in defending the intestine from enteric pathogens. The microbiota inhibits pathogen growth through three main mechanisms: direct inhibition of pathogen growth, nutrient depletion of the surrounding environment, and through stimulation of the innate and adaptive immune responses of their host (Stecher & Hardt, 2011). Bacteria can directly inhibit the growth of others by releasing toxic metabolic byproducts, such as acetate or butyrate, or they might release

antimicrobial peptides called bacteriocins (Stecher & Hardt, 2008). In addition, other members of the gut microbiota in general deplete their environment of necessary nutrients such as oxygen or carbohydrates in mucins as is the case with certain strains of *E. coli* (Stecher & Hardt, 2011). This can prevent other, potentially pathogenic bacteria from occupying this niche and causing significant disease in a process called competitive exclusion.

More interestingly, perhaps, is the idea that byproducts of the microbiota, such as released factors from the cell walls of these bacteria, can support the maturation of immune tissues (Stecher & Hardt, 2008). However, it is not clear whether specific species are responsible for these functions, or if it is a general byproduct of having these microbes present at all. A greater understanding of the microbiota can, therefore, have significant impacts on human health overall.

The Zebrafish Model

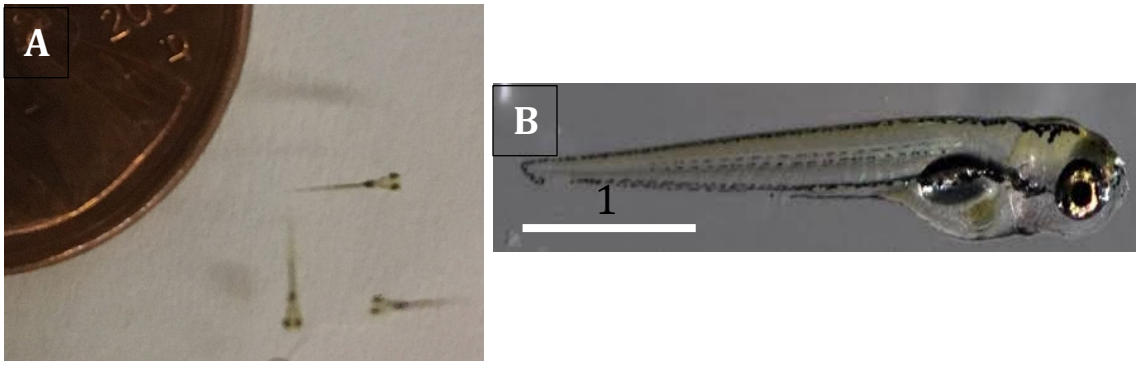


Figure 1: Several zebrafish larvae approximately seven days post fertilization (dpf) pictured next to a penny to show relative size (A). A close up of a larva of similar age next to a scale bar (B). Images courtesy of Cathy Robinson and Hampden-Sydney College respectively.

Zebrafish are good model organisms for studying host-microbe interactions for a variety of reasons. Their high fecundity allows hundreds of eggs to be collected at one

time from a single tank of adult fish, providing many offspring of similar age and genomic composition to study at once. In addition, because the insides of the eggs are sterile, hundreds of zebrafish can be easily derived germ-free, or in the absence of microbes, by surface sterilization of the eggs and rearing in a sterile environment. This allows the microbial composition of these communities to be carefully controlled and manipulated, using well-defined microbial isolates (Melancon et al., 2017). The added benefit of these defined microbial isolates is that they can be maintained in laboratory culture and many of their genomes have been sequenced. Genome sequencing and assembly is an expensive and time consuming process, but when completed it facilitates mutation detection, and can make it easy to compare genes across bacterial species and find similarities and differences that can aid the researcher. In addition, the transparency of zebrafish larva allows for monitoring of both host and microbial cells *in vivo* (Jemielita et al., 2014), but this particular benefit will not be explored in this project.

Evolution in Bacterial Populations

One of the fundamental tenets of biology is that populations of organisms evolve (Freeman, 2011). Charles Darwin and Russell Wallace independently formulated the theory of evolution by natural selection in the 1850s and Darwin published his book *On the Origin of Species by Means of Natural Selection* in 1859. In this work, he broke down his theory of evolution into four postulates. First, individual organisms in a population vary in traits they possess. Second, some of this variation in traits is heritable, which means that there is a tangible difference in the genomes of these organisms which can be passed down to their offspring. Third, only some of the offspring produced survive long enough to reproduce depending on the fourth postulate:

that the individuals that best survive and reproduce are not random. Instead natural selection occurs over many generations when individuals with certain traits produce more offspring than individuals with other traits (Freeman, 2011). These adaptive mutations increase the fitness of the individual compared to the population and, over many generations, can shape the formation of new species. The environment plays a role in which traits are favored in these evolving populations, as well as many other factors. Evolutionary biology uses this guiding theory to investigate how the diversity of species evolved on earth. Because all species on earth evolved from a common, unicellular ancestor, it is useful to examine how bacterial populations evolve asexually to understand evolution on a unicellular scale.

Bacteria reproduce by copying their own genome, which has a single copy of each gene (haploid), and dividing through the process of binary fission. Errors sometimes occur in this gene replication, and because each bacterium only has one copy of the gene, it will immediately impact the biology of the cell. Many mutations are possible, from gene loss, which can either completely remove the product protein of a particular gene from the cell, or a change in a single nucleotide could render a protein ineffective. More rarely, this random point mutation might cause a change in protein that makes it more effective in the cell, or entire genes may be duplicated. In addition, there are mutations that can affect gene expression rather than the protein itself. These could affect the sequence that recruits RNA polymerase directly or in the binding sites for proteins that either recruit or prevent the RNA polymerase from binding and making the transcript that will later serve as the recipe for protein production. On an individual level, these mutations might be beneficial, neutral, or deleterious to the particular

bacterium in a population. When these heterogeneous individuals are faced with a change in environment, the heritable variation provides the raw material for natural selection with the fittest individuals surviving to propagate (Brock, 2012). This eventually leads to a population dominated by individuals that possess the traits that allow them to survive longer, and thus reproduce more than others, as described by the classical model of the evolution of asexual organisms (Rosenzweig et al., 1994).

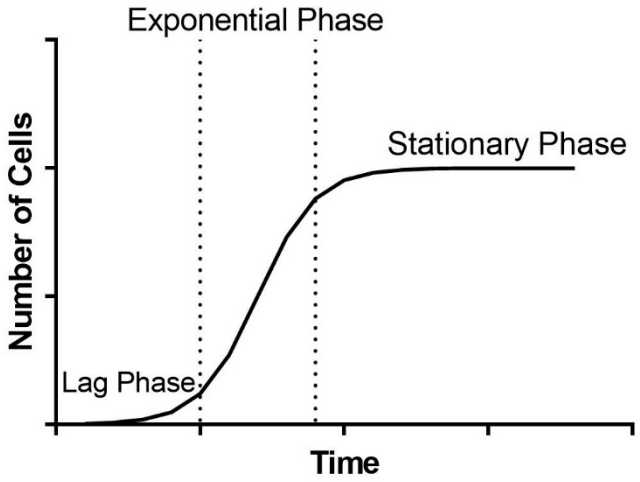


Figure 2: A typical bacterial growth curve in rich media.

Bacterial growth varies due to nutrients present, and sometimes there are more lags phases, especially if the bacterium is switching from one energy source to another (eg glucose to lactose). In addition, after stationary phase there is a death phase once the nutrients in the media are exhausted.

Jacques Monod, in particular, contributed to the study of bacterial evolution through his work with the chemostat in the 1950s (Schaechter, 2015). In the typical laboratory, a bacterial culture will grow exponentially, with one cell dividing into two and then four and then eight, until the available resources are exhausted or a build-up of waste products becomes toxic. There is then a period of time where the population

maintains viability but no longer reproduces until the population numbers decline as cells die (Figure 2). Monod used a chemostat, which continually introduces fresh nutrients to a culture while removing wastes, to allow cultures to perpetually grow exponentially. This allowed him to reproduce in the lab one of the necessary prerequisites of evolution: a population that is maintained for the many generations required for natural selection to occur.

Experimental Evolution

Experimental evolution refers to the practice of studying evolving populations under controlled conditions (Hoang et al., 2016). Monod's work on continuous culture paved the way for the field of experimental microbial evolution. Seminal work includes that of Novick and Szilard, Bryson and Szybalski, and Atwood, Scheider and Ryan (Adams & Rosenzweig, 2014). Each of these groups used different methods to continuously culture bacteria, namely the chemostat, turbidostat (similar to chemostat but also uses turbiditiy to monitor how often dilutions are made), and serial dilutions (moving a small sample of a culture to a flask with fresh media at regular intervals) respectively. They all found that there were certain mutations that were selected for during this extended growth, as predicted by the theory of evolution (Adams & Rosenzweig, 2014). Subsequent work was focused on the analysis of these mutants that were selectively favored in each growth method with a heavy focus on how environmental factors affect enzyme activity. More recently, experimental evolutionary techniques have been used to study a variety of processes including adaptations to particular nutrient environments, (Rosenzweig et al., 1994), the effects of environment on the evolution of particular traits (Bjorkman et al., 2000), and even how a commensal

bacterium evolves within a host (Barroso-Batista et al., 2014). These papers show how valuable experimental evolution techniques are to understanding microbial interactions with their environment both within and without a host.

In the Guillemin lab, an experimental evolution techniques model system has been developed to investigate how a symbiotic bacterium evolves to the host gut. However, the specific impact of environmental adaptation on the zebrafish gut is less explored. Previous work has found that decreasing motility and chemotaxis of a symbiont in its environment significantly decreases host colonization (Stephens et al., 2015). However, the specific function of this bacterial motility is unknown. Are motile strains more available in the water column, increasing their sampling rate in order to colonize the gut more effectively? Results from experimental evolution might indicate that this plays a factor, since evolved strains that are better at colonizing the host also grow to greater densities in the environment. Growing to these higher densities is remarkable, because there are very few nutrients in the fish water environment, especially in a germ-free one like for these experiments. Perhaps the host is releasing a factor that allows its symbionts to grow to greater densities, or chemotax to the host. *Aer01* in particular can grow in the fish- conditioned embryo media (FC-EM) water environment, without the host, indicating that there is a factor in this media that is affecting symbiont growth. I predict that as the symbiont adapts to this FC-EM environment, it will develop mutations that increase its environmental colonization fitness, which will directly translate to increases in host colonization fitness. The purpose of this project is to identify changes in the symbiont's genome can help to piece together this environmental impact of host-colonization.

Methods

Bacterial Strains

For this study, I used a bacterial strain of *Aeromonas veronii,* ZOR0001 (NCBI accession number: PRJNA205571). Ancestral and reference strains for my evolution competitions had been previously fluorescently-tagged with dTomato (dT) or superfolder green fluorescent protein (GFP) using a Tn7 transposon system as described in Wiles et al. (2016). This method resulted in the incorporation of a cassette with the *dTomato/GFP* gene and a gentamycin resistance gene in the chromosome at a specific target location. Mutations of interest (clean deletion or point mutation) were generated in the untagged wildtype using an allelic exchange protocol (described in Wiles et al. (2017)). Each time strains were grown from cryo-archived isolates, they were started in 5 mL tryptic soy broth (TSB) and grown overnight at 30°C with shaking.

Gnotobiotic Zebrafish Derivation and Husbandry

All experiments involving zebrafish were conducted following standard protocols and procedures approved by the University of Oregon Institutional Care and Use Committee. Wildtype (AB x Tu strain) fish were used for all experiments. These fish were maintained using the protocols previously described (Westerfield, 2007). Gnotobiotic zebrafish were derived using the techniques described by Melancon et al. (2017) and the larvae were not fed during the course of the experiment. For all experiments involving zebrafish larvae, the fish were inoculated with bacterial cultures at 4 days post fertilization (dpf). At 7 dpf, fish were euthanized with tricaine (Western Chemical, Inc.) following approved procedures, mounted in sterile 4% methylcellulose

solution (Fisher), and the intestines were removed by dissection as described in Milligan-Myhre et al. (2011). These intestines were used for enumeration of colonization through serial dilution, and plating on TSB.

Passaging in Fish-Conditioned Water

Evolution passaging was initiated using dT-tagged *Aer01* grown overnight from a cryo-archived isolate. Overnight culture was pelleted (8,700 rcf, 2 min), washed in sterile embryo media (EM) then diluted to a CFU/mL count of approximately 10^3 in triplicate samples of 2mL fish-conditioned embryo medium (FC-EM) to begin passaging. FC-EM was collected from flasks of hatched, germ-free larvae 4dpf and filter sterilized with a 0.2 um filter-tipped syringe. Cultures were allowed to grow overnight at 30°C with shaking. After 24 hours, cultures were diluted 1:100 in fresh FC-EM to begin the next passage and a sample was taken and serial dilution plated on Tryptic Soy Agar (TSA) to measure population density. This procedure at 24 hours was repeated for 26 passages for all lines.

Every four passages, a sample of the whole population of evolved isolates from each line was suspended in 25% glycerol directly from the 24 hour culture and stored at -80°C. Isolates were then colony purified directly from the freezer stocks of whole populations from passages 4, 8, 12, 16, 20, and 24. Cells were streaked out on TSA plates for isolation, then incubated at 30°C. The following day, isolated colonies were transferred into 5mL TSB and allowed to grow for approximately 5 hours. Then a sample of each isolate in TSB was suspended in 25% glycerol and stored at -80°C.

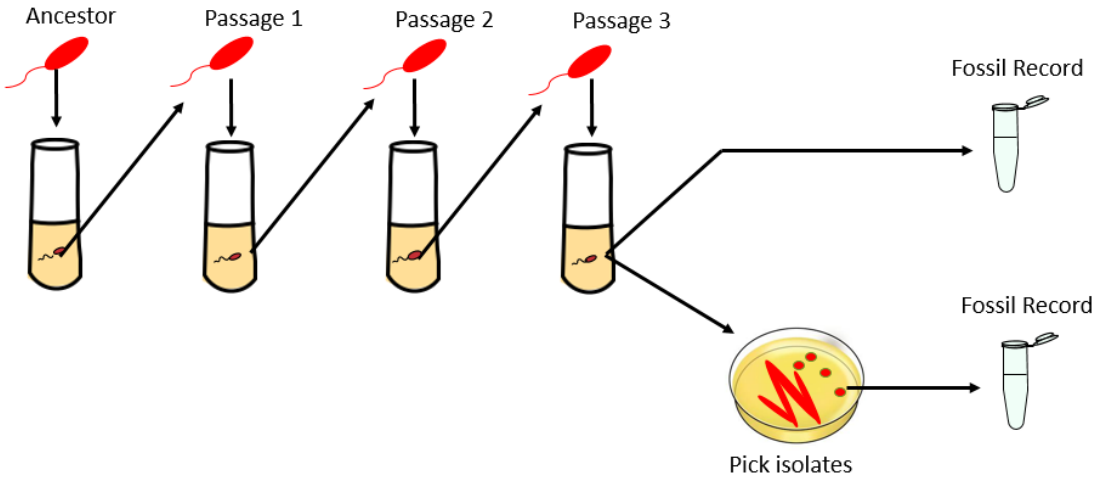


Figure 3: Diagram of Evolution Passaging.

Passaging of dTomato tagged *Aer01* strain in FC-EM. Every four passages the entire population was cryo-preserved in 25% glycerol and streaked for isolation. There was no other mixing of rich and minimal media.

Phenotypic Assays for Adaptation

Rich Media Growth Curves

Overnight cultures of selected isolates were diluted 1:100 in TSB and placed in triplicate in a 96 well polystyrene tissue culture plate with TSB blanks. The plate was then placed in the FLUOstar Omega Microplate Reader (BMG Labtech, Ortenberg, Germany) at 30°C for >15 hours with shaking with absorbance readings taken at 600nm every 20 minutes.

In vitro Competitions

Strains were first grown overnight in TSB culture, inoculated into 2mL FC-EM, to a final density of approximately 10^3 CFU/mL. The following day the ancestor or evolved isolate and GFP-tagged reference strain were mixed (ratio of 1:5, ancestor/evolved:ref) and the resulting competition mixture was diluted 1:100 in FC-

EM. The starting ratio was measured by serial dilution and plating, and the competition culture was placed at 30°C, and the final ratios were measured at 24 hours, again by serial dilution and plating. The competitive index of each experiment was calculated as described in Equation 1.

$$\text{Competitive Index} = \frac{\left(\frac{\text{evolved or ancestor}}{\text{reference}}\right)_{\text{final}}}{\left(\frac{\text{evolved or ancestor}}{\text{reference}}\right)_{\text{starting}}}$$

Equation 1: Competitive Index calculation.

This calculation allows us to compare starting ratios to final ratios to determine whether one strain has a competitive advantage in growth or colonization in a particular environment compared indirectly to the ancestral strain. We cannot compare directly in competition because evolved isolates and the ancestral strain have the same dTomato tag and thus a direct competition would be inconclusive. A competitive index of 1 means that there was no difference in growth between the starting and final ratios, and implies that one strain does not have a competitive advantage over the reference.

In vivo Competitions

Competitions conducted in fish used overnight TSB cultures of reference and ancestral or evolved isolate strains. The cultures were spun down (8,700 rcf, 2 min), washed with sterile EM, spun again and resuspended in the original volume of sterile EM. Once the cultures were washed, they were combined in 1:5 ratios of evolved/ancestral strain to a gfp-tagged reference strain. Next 10 µL of these competition mixtures were used to inoculate flask of 4 days post fertilization germ-free derived zebrafish larvae (each flask contains 15 fish and 15ml EM). A sample of the initial water concentrations were spread plated at this time. At 7 dpf, larvae were sacrificed and dissected as previously described and guts were placed in 500 µL sterile EM with 0.5 mm zirconium oxide beads (Next Advance, Averill Park, NY) and blended

(Power 4, 30 seconds) using a bullet blender tissue homogenizer (Next Advance, Avrill Park, NY). CFU/gut were then measured using serial dilution and plating. A sample of the final flask water was also collected and plated at this time. Competitive indexes were determined as described above (Equation 1).

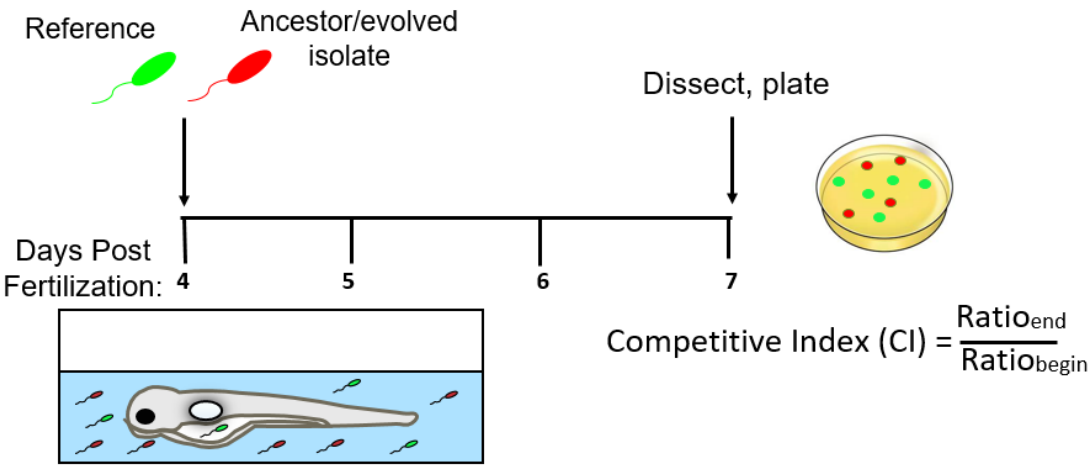


Figure 4: Schematic for host-colonization competitions.

Each competition assay uses a flask of 15 fish, from which eight were sampled and their guts examined. Competitive indexes were compared using the final gut ratio and the initial water ratio, because there should be no competition bacteria in the gut at the moment of inoculation.

Biofilm Assay

Biofilm formation was assayed using methods similar to those previously described (Merritt, Kadouri, & O'Toole, 2005). The strains used in these assays were colony-purified evolved isolates from the evolution passaging, and mutants generated from the WT strains. These were grown from cryo-preserved stocks (as described above) and assays were done in biological triplicate. Controls were sterile TSB incubated and stained like the bacterial culture wells. 200µL of 1:100 dilutions of overnight TSB cultures were incubated for 24 and 48 hours at 30°C in a 96-well

polystyrene tissue culture plate. To quantify biofilms, first supernatants were discarded by aspiration then washed with deionized H2O at 150rpm for 10 minutes. Next, the supernatant was discarded again and 200µL of 0.1% crystal violet was added to each sample well and allowed to stain for 10 minutes at 150rpm. The stain was removed and wells were washed with ddH2O until was clear. Finally, 200µm of 100% Ethanol was used to dissolve the stain. The ethanol solution was transferred to fresh wells and read at OD540.

Motility Assay

Plates for this assay were made with TSB, with a final concentration of 0.2% agar (VWR Life Science AMRESCO Agarose). The mixture was boiled in the microwave on high for 1 minute until all of the agarose was dissolved. The solution was then placed on a rotating plate to cool for approximately 10 minutes. After this time, the mixture was transferred to sterile petri dishes and allowed to dry for 1 to 2 hours partially uncovered near a flame. Once cured, the plates were inoculated with 1µL from overnight TSB cultures with 3 isolates and 1 ancestor control per plate. The plates were incubated for 4 hours at 30°C then zones of growth measured and imaged.

Whole Genome Sequencing

To prepare samples for sequencing, I started overnight cultures as described in "Bacterial Strains" in TSB of isolates from passage 12, lines two and three, as well as isolates from passage 20, all lines. I also prepared whole population samples from all lines for passages 8, 12, and 20 in this way and a sample of the ancestral dT-tagged *Aer01* strain. I then purified genomic DNA from these samples using the Progmega

Wizard Genomic DNA Purification Kit and eluted the DNA using molecular grade H2O and stored at 4°C. I used these purified genomic DNA samples to prepare a Next Generation Sequencing Library for Illumina sequencing using the Nextera DNA Flex Library Prep Kit.

Once samples were sequenced, Breseq (Deatherage et al., 2014) was used to align the sequencing reads to the reference genome and predict mutations and possible genomic rearrangements.

Results

Experimental Evolution Passaging

Over the course of twenty-six passages of the dT-tagged ancestor, I saw the population density of all lines increase in Fish-Conditioned Embryo Media (FC-EM). While line one initially decreased greatly compared to the other lines, on average all lines increased in density over the course of the passaging events (Figure 3a). In particular, I saw a 10-fold increase in populations by passage 6, so I selected the closest cryo-preserved stock for further testing (passage 8). I chose two other time points for further investigation, namely passage 12 and passage 20 (Figure 3b). Passage 12 was remarkable because it was the earliest cryo-preserved passage where all three lines had increased greatly in population density, signifying perhaps that these lines were more adapted to the environment at this point without accumulating too many background mutations (changes from the ancestral strain that do not change the growth phenotype). In addition, I selected passage 20 for further testing because all three populations reached their peak density at this passage.

After each passage was preserved, I streaked the whole population for isolation from the freezer stock and selected three isolates per passage for further analysis. Evolved isolates have the potential to help us identify specific mutations that could not only help the bacterium to grow in its FC-EM environment, but also could impact fish colonization. From this point forward, I will refer to whole populations by their passage number and line (eg. 8-2 for passage 8, line 2) and I will refer to evolved isolates by their passage number, line, and isolate letter (eg 12-2A for passage 12, line 2, isolate A).

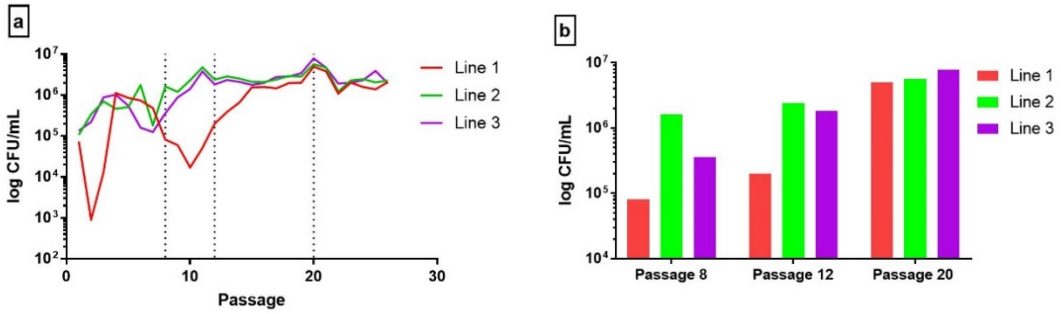


Figure 5: *Aer01* passaged in FC-EM increases in population density.

Figure 5a shows population density at the time of passaging, 24hrs after inoculation. Populations density increased in two of the three lines approximately 10 fold once by passage 8 (dashed line 1) and again by passage 12 (dashed line two) as shown in the increase in colony forming units per milliliter. The third dashed line shows the highest density in all three lines that occurred during passaging. Figure 5b directly compares population density at these specific passages.

Fitness Changes in FC-EM and Fish Gut Colonization

Evolved isolates from passage 12 consistently out-competed the ancestor in FC-EM (Figure 6). A variety of factors could be influencing this change. The evolved isolates could be better able to persist in FC-EM until the completion of the competition at 24 hours. Or, the growth of the isolate could be different in this media, either higher densities as seen in Figure 3 or the log phase of the bacterium's growth could be affected in some way. However, there was no significant difference across evolved isolates from all three lines in growth in rich media (Supplemental Figure 1).

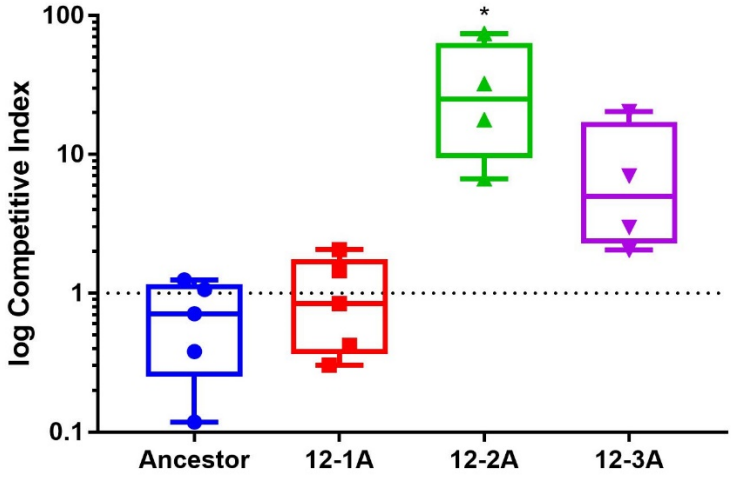


Figure 6: Passage 12 FC-EM isolate competitions show significantly different levels of fitness.

Isolate 12-2A had a significantly higher competitive index compared to the ancestor (ANOVA, P=0.0218). 12-3A did not have significantly higher competitive indexes (P=0.864) but there was still an increase not seen in the ancestor or 12-1A. Dotted line at CI=1 to show where there is no difference between starting and ending ratios.

In order to narrow down potential candidates for sequencing, I selected evolved isolates for competitions in larval zebrafish. I chose candidates from passage 12 because this passage was the earliest that there was a large increase in population density (Figure 3). Interestingly, I found that high competitive indexes in the flask water, most similar to the FC-EM environment that the isolates had been passaged in, did not necessarily translate to a greater gut colonization ability like in passage 12-2A.

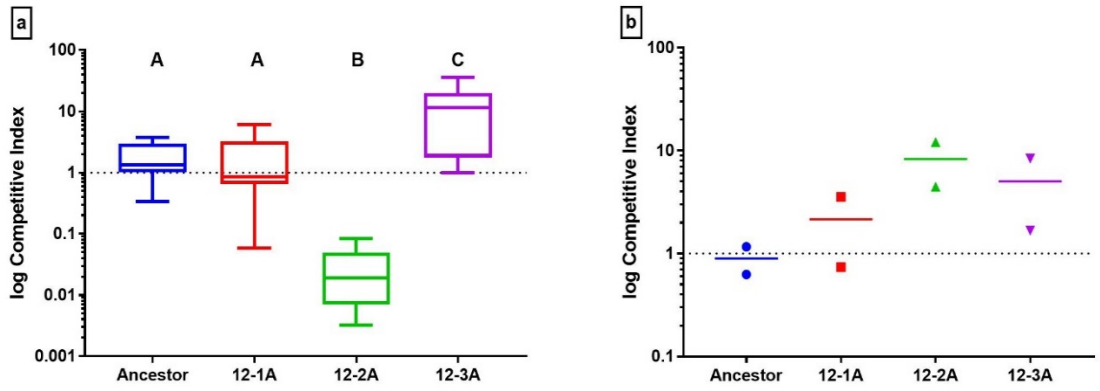


Figure 7: Passage 12 Isolates have significant changes in host colonization.

Figure 7a: Both isolates 12-2A and 12-3A had significant differences in competitive index (ANOVA, $P<0.0001$). Figure 7b: While isolates from most lines significantly out-competed the ancestor in the flask water, this did not always translate to an increased gut colonization ability.

Instead, the isolate that had the highest CI in the flask water was significantly worse at colonizing the fish gut compared to the ancestor, contrary to my hypothesis.

In order to unravel some of these changes that were occurring as the populations in the three lines adapted to their FC-EM environment, I sequenced the genomes of selected whole populations and single isolates using Illumina Next Generation Sequencing (NGS). I selected whole populations from all three lines in passage 8 and 12 to hopefully track newly arising mutations across each population. I selected single isolates for NGS genomic analysis from those that had a significant change in either *in vitro* or *in vivo* competitions. Thus, I selected evolved isolates 12-2A, 12-2B, 12-3A, 12-3B, 20-1A, 20-1B, 20-1C, 20-2A, 20-2B, 20-2C, 20-3A, 20-3B, 20-3C. Illumina sequencing uses the chunks of genome from each sample with specific barcodes and tells me the identity of each base in each of the sequences. However, it is the most

accurate with sequences that are around 100 basepairs long (each is called a read), and it is less accurate in sequencing long strings of the same base (eg TTTTTT). For a genome of 4 million base pairs (4 Mbp) like *Aer01,* there are at least 400,000 reads, and many more to find how each of these interact for each genome. For my samples, I had, on average, 300 reads that overlapped for each point in the genomes that I was interested in. To work with this mountain of data, I used the software Breseq to align each sequence read with the reference wildtype genome and look for differences, which are predicted potential mutations or predicted genomic rearrangements (Deatheridge et al., 2014). One caveat with this method is that there are more predicted mutations in regions of the genome that have many repeats, or near repeats, and thus predicted mutations should be analyzed separately to make sure that they are not in these regions.

There were only two mutations of interest that occurred both across many isolates and whole populations and occurred with enough coverage to have high confidence (Supplemental Table 1). However, due to the restraints of NGS sequencing and assembly, regions with large repeats often return many suspected mutations due to the difficulty of properly mapping nearly identical sequences to the genome. This eliminated one of the two mutations of interest, the Leukotoxin gene *IktA* is large and has many repeating sequences, so it is unlikely that there is a true mutation in this region. Thus, I selected the Type 1 Secretion System gene *prsE* for further analysis by introducing this mutation into the wildtype *Aer01* genome.

There were also some potential genomic rearrangements in isolates that had phenotypic differences from the ancestor. In particular, the isolate which outcompeted the reference in fish, 12-3A, did not have any predicted mutations, only predicted

genomic rearrangements in genes affecting motility among others. However, these rearrangements require more sophisticated analysis of the genomic data, which is why I did not follow up initially. These should be a target of further research in the future.

Type I Secretion System Protein prsE Mutations

Mutations in the *prsE* gene in evolved isolates that grew to significantly higher densities in the FC-EM included a single nucleotide insertion of a thymine at position 666 in the gene also known as a point mutation. This insertion resulted in an early stop codon and results in a protein that is approximately half its original size I hypothesized that this truncated protein was functionally similar to a complete deletion of *prsE.* In order to test this hypothesis, I generated the point mutation as well as a *prsE* deletion or knockout in the wildtype *Aer01* strain. I found that there was no significant difference in growth in rich media between these generated mutants and the wildtype (Supplemental Figure 2). However, in FC-EM, both the knockout and the point mutant reached higher densities than the ancestor, and also beat it in competition (Figure 8). A functioning *prsE* seems to be performing an inhibitory function in *Aer01* growth in minimal media such as FC-EM.

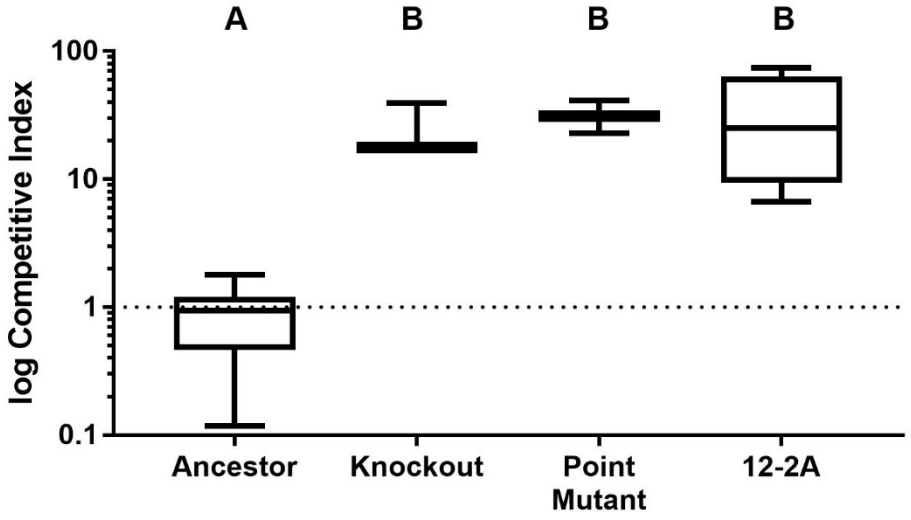


Figure 8: Mutations in *prsE* increase competitive fitness in the fish environment.

The *prsE* knockout, *prsE* point mutant and evolved isolate significantly outcompete the ancestor in FC-EM, but do not differ in colonization ability among each other (ANOVA, P=0.0083). This indicates that loss of *prsE* might aid colonization in low nutrient environments.

In contrast, there was no significant difference in zebrafish gut colonization for either the *prsE* point mutant or the knockout (Figure 9). This is unexpected because isolates with this mutation, like 12-2A were significantly worse at colonizing the gut (Figure 7). These results pointed to some other mechanism, perhaps unrelated to the *prsE* induced increases in growth density, which was causing such a significant decrease in colonization ability. Previous researchers found that *prsE* was important in biofilm formation in *Rhizobium leguminosarum* (Russo et al., 2006). However, I found no significant difference between my evolved isolates with *prsE* mutations, generated *prsE* mutants, or the wildtype (Supplemental Figure 3).

Another possible explanation has to do with cell motility. Other researchers have found that decreased motility can decrease zebrafish gut colonization of different bacterial strains (Wiles et al., 2016). In addition, a common outcome of evolution experiments, especially with a motile bacterium in a shaking environment, is loss of motility (Zhou et al., 2013). In addition, Breseq predicted rearrangements affecting genes involved in motility in the same isolates that had *prsE* mutations. Thus, I conducted a motility assay using rich media swim plates. I found no significant difference between the wildtype and *prsE* mutants (Figure 5c & d). In contrast, there was significantly reduced motility in evolved isolates with *prsE* mutations, which might account for the changes in host colonization ability (Figure 5a &b).

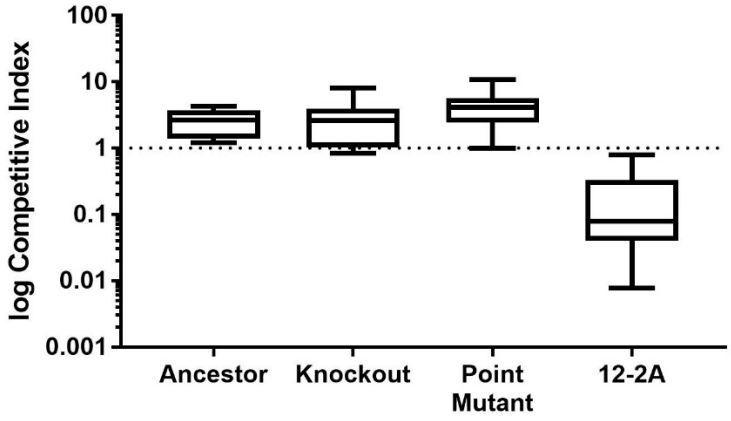


Figure 9: *PrsE* mutants have no change in host colonization compared to the ancestor.

The higher growth densities of the *prsE* mutants does not translate to increased host-colonization ability, instead neither the knockout or point mutant is significantly different than the ancestor (ANOVA, $P > 0.05$). This suggests another mechanism is responsible for the significant decrease in host-colonization ability in isolate 12-2A (ANOVA, $P= 0.0038$).

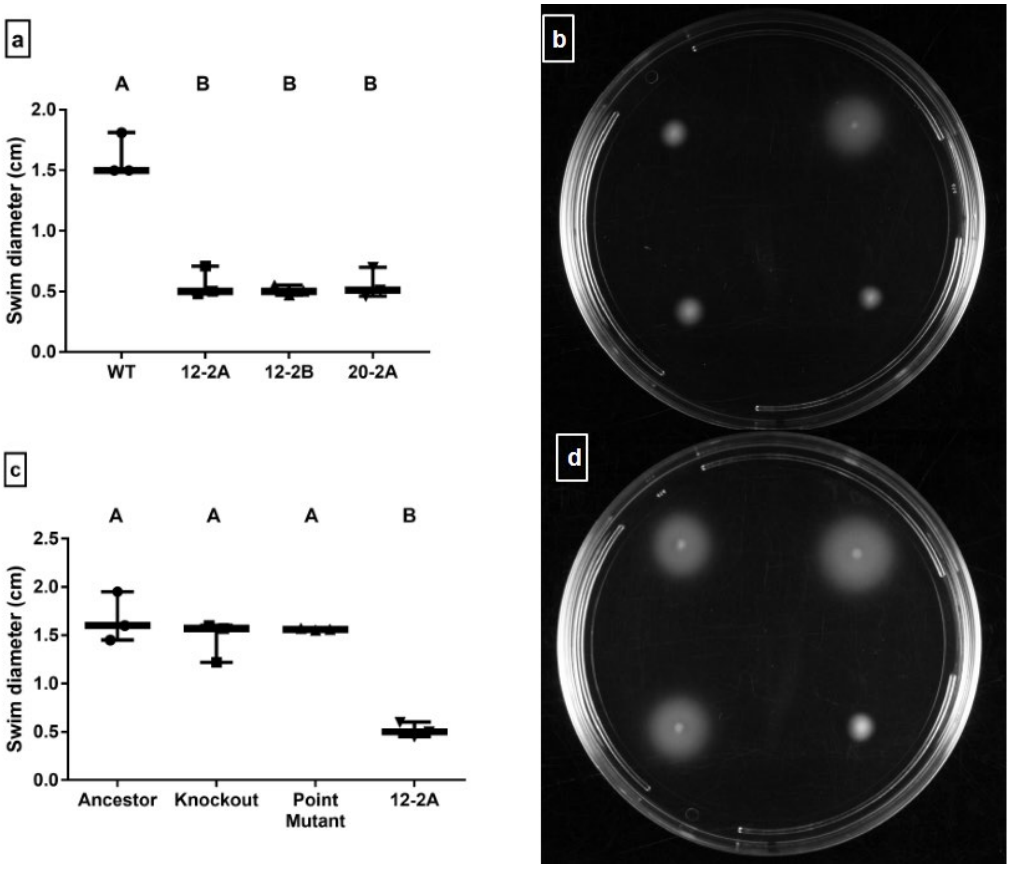


Figure 10: Evolved isolate motility in 0.2% agarose rich media swim plates.

Figure 10a shows a statistically significant difference between the wildtype and evolved isolates above in biological triplicate while there was no significant difference in motility between the isolates (ANOVA, $P<0.0001$). Figure 10b shows an example of the swim plate with the arrangement of isolates clockwise from the upper right corner: WT, 12-2A, 12-2B and 20-2A. 10c compares generated *prsE* mutants to an evolved isolate and the WT, while Figure 10d shows a swim plate inoculated clockwise from upper right: WT, 12-2A, PM, KO.

Discussion

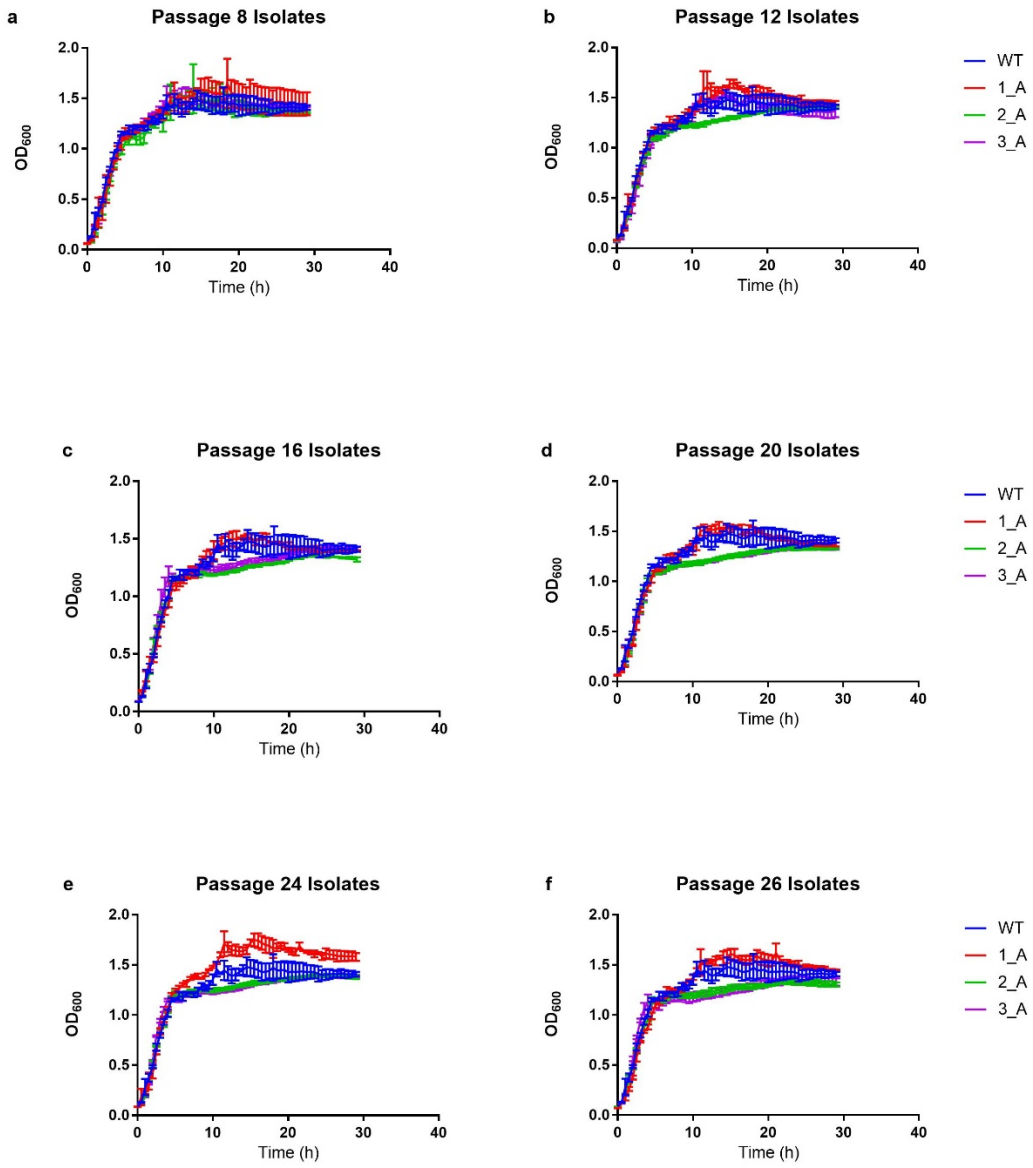
Throughout the course of this project, I found that experimental evolution is a viable way to uncover new phenotypes in a symbiotic zebrafish bacterium. Not only were there clear changes in growth ability within as few as eight passages, there also was a variety of potential genes of interest for further investigation. In particular, loss of function of the Type I Secretion System membrane protein *prsE* seems to help increase growth ability in *Aer01* in a nutrient-poor medium like FC-EM. I hypothesized that increasing bacterial growth in the environment would directly increase the bacteria's gut colonization ability. However, I found that merely increasing bacterial populations in the fish environment did not guarantee an increase in gut colonization, instead there was no change in gut colonization when we removed *prsE* all together in the absence of other changes. This indicates that the evolved isolates with *prsE* loss of function mutations had other genetic changes that made them significantly worse at gut colonization.

One potential mutation that might be responsible for this colonization phenotype could result in changes in motility. I saw that there was significantly decreased motility in each of the evolved isolates, mainly from line 2, which also had decreased fish colonization ability (Supplemental Table 2). Other researchers in the field have also found that motility is important for zebrafish gut colonization. In addition, changes in chemotaxis have been indicated to change colonization. More in-depth analysis of genes required for these phenotypes is warranted.

Adaptive evolution experiments have the potential to unlock many of the mysteries of host-bacterial interactions by allowing researchers to find genes important

for these interactions without previous knowledge of their functions. In fact, there are many more possible candidates for sequencing and analysis within my cryo-preserved isolates. Future work could continue this vein of investigation, and help contribute to our basic understanding of host colonization dynamics.

Supplementary Figures and Tables



Supplemental Figure 1: Rich media growth curves of all evolved isolates.

Replicate A from all of the lines at each preserved passage was grown in rich media for over 24 hours in triplicate and sampled every 30 minutes. There was no significant difference in rich media growth across the evolved isolates and the ancestral strain.

Gene	Mutation	Affected Whole Populations	Affected Isolates
prsE	+T at position 2080420	8-2, 12-2	12-2A, 12-2B, 20-2A, 20-2C, 20-3A
IktA	C→T at position 167559	12-2,	12-2A, 12-2B, 20-2A, 20-2C, 20-3A,

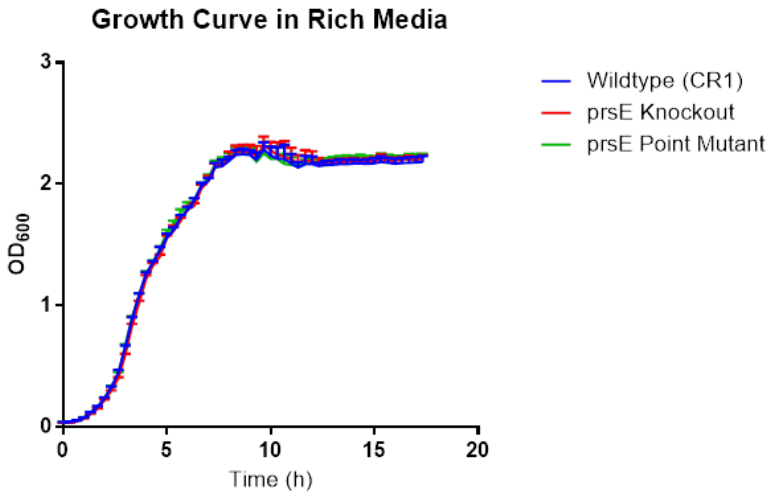
Supplemental Table 1: Breseq-predicted mutations and affected groups.

These mutations in genes of interest were selected using Breseq analysis. In order to appear on this table, read depth needed to be at least 300 and mapped reads must be greater than 75%.

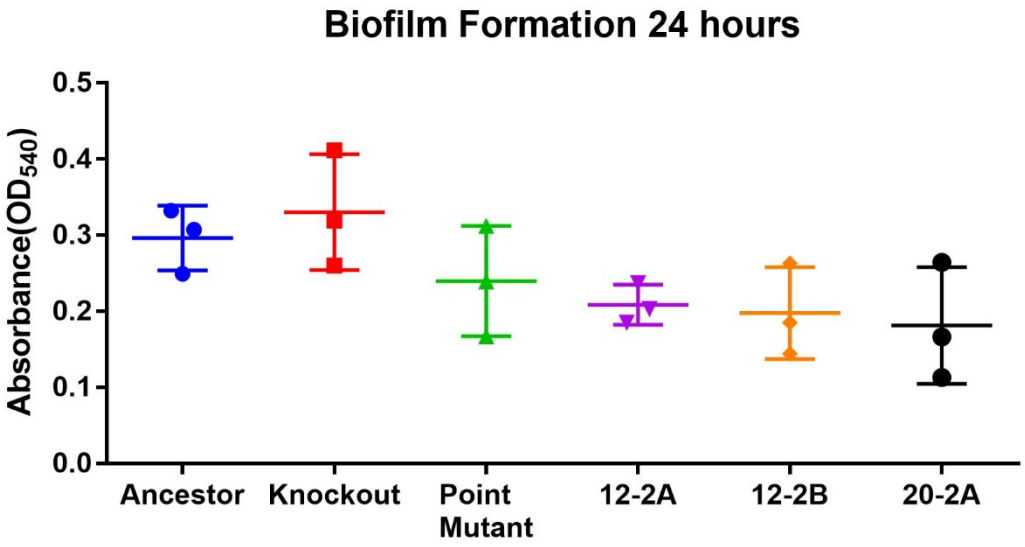
Gene	Gene Product	Affected Position	Affected Isolates
TnpB	Methyl-accepting chemotaxis protein transposase	275264	12-2A, 12-2B, 12-3A, 12-3B, 20-1B, 20-2B, 20-1C, 20-2C, 20-3A, 20-3B, 20-3C
FlgA	Flagellar basal body P-ring biosynthesis protein	912013	12-2A, 12-2B, 20-2A, 20-2B, 20-2C, 20-3A, 20-3B, 20-3C
CsrD	RNAase specificity factor	2117957	20-1A, 20-1B
yccM_2	Putative electron transport protein	4457713	20-3C

Supplemental Table 2: Breseq-predicted genomic rearrangements in isolates.

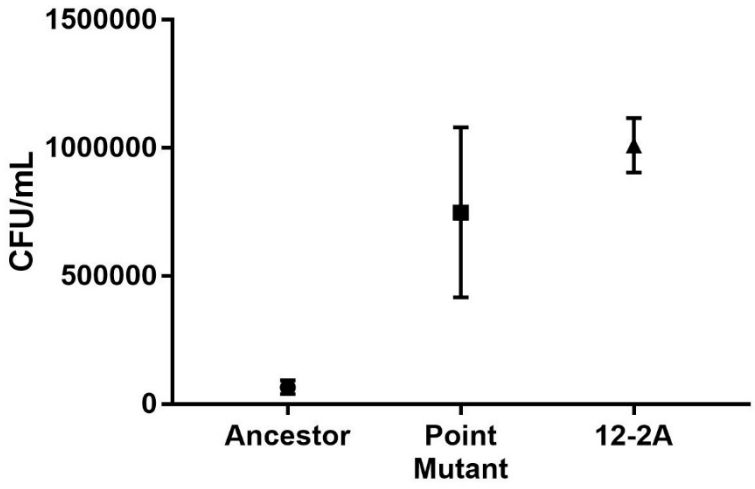
These genomic rearrangements, especially in motility genes, such as the flagellar basal body P-ring biosynthesis protein gene, might decrease host colonization.



Supplemental Figure 2: There is no change in growth fitness in rich media with mutations in prsE expression.



Supplemental Figure 3: Biofilm formation in WT, evolved isolates, and *prsE* mutants.



Supplemental Figure 4: *PrsE* point mutants have increased densities in FC-EM.

The *prsE* Point Mutant and evolved isolate grow to significantly higher densities in FC-EM compared to the Ancestor over 24 hours. The above graph shows growth as calculated by final CFU/mL – initial CFU/mL. There were two trials using two biological replicates.

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