The evolution of virulence in *Pseudomonas aeruginosa* during chronic wound infection

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ABSTRACT

Opportunistic pathogens are associated with a number of widespread, treatment-resistant chronic infections in humans. As the pipeline for new antibiotics thins, virulence management presents an alternative solution to the rising antimicrobial resistance crisis in treating chronic infections. However, the nature of virulence in opportunists is not fully understood. The trade-off hypothesis has been a popular rationalization for the evolution of parasitic virulence since it was first proposed in the early 1980's, but whether it accurately models the evolutionary trajectories of opportunistic pathogens is still uncertain. Here, we tested the evolution of virulence in the human opportunist *Pseudomonas aeruginosa* in a murine chronic wound model. We found that in a serial passage experiment where transmission potential is no longer an epidemiological restriction, virulence does not necessarily increase as is predicted by the trade-off hypothesis, and in fact may evolve in different directions. We also assessed *P. aeruginosa* adaptations to a chronic wound after ten rounds of selection, and found that phenotypic heterogeneity in *P. aeruginosa* is limited in chronic wounds compared to heterogeneity seen in cystic fibrosis (CF) infections. Using next-generation sequencing, we found that genes coding for virulence factors thought to be crucial in *P. aeruginosa* pathogenesis, acquired mutations during adaptation in a chronic wound. Our findings highlight that (i) current virulence models do not adequately explain the diverging evolutionary trajectories observed during *P. aeruginosa* chronic wound infection, (ii) *P. aeruginosa* phenotypic heterogeneity is less extensive in chronic wounds than in CF lungs, (iii) genes involved in *P. aeruginosa* virulence acquire mutation in a chronic wound, and (iv) similar adaptations are employed by *P. aeruginosa* both in a chronic wound and CF lung.

INTRODUCTION

Opportunistic pathogens, those that only cause disease when host immune defenses are weakened, are responsible for a number of chronic, difficult-to-treat human infections, such as certain skin, respiratory, and urinary tract infections. Common problematic human opportunists include *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Candida albicans*, *Klebsiella pneumoniae*, *Serratia marcescens*, and *Acinetobacter baumannii*. While chronic infections caused by opportunists are prevalent, the complex nature of their virulence remains elusive. Investigating the dynamics of virulence in chronic infections is of rising interest as researchers turn to novel treatments, such as anti-virulence drugs, to combat rapidly increasing antimicrobial resistance [1-4]. Yet, there are two core questions to which the answers are still unclear: How do opportunists evolve virulence, and are patterns of evolution predictable?

Currently, there are four main hypotheses for the evolution of pathogenic virulence, where virulence is attributed to either (i) new host-parasite associations, (ii) evolutionary trade-offs, (iii) coincidental selection, or (iv) short-sighted evolution [5-7]. Of these, the trade-off hypothesis has received much attention and has been extensively tested and debated. First proposed in 1982 by Anderson and May, the trade-off hypothesis replaced the 'conventional wisdom' of the time– that parasites should evolve towards avirulence or commensalism [8]. Though there have been many variations of the trade-off hypothesis, the most widely studied trade-off is between virulence and transmission [8-13]. This interpretation of the hypothesis assumes that virulence and transmission are linked via within-host replication. In this model, parasites adapt to increase both their abundance and transmission potential, and in doing so inevitably exploit their host, at the cost of reducing infection duration and increasing the likelihood of host death. As parasites cannot simultaneously increase transmission and prolong infection according to this framework, they must maximize their overall reproductive fitness by trading off between the two, selecting for intermediate virulence [7, 11, 13].

While the trade-off hypothesis has been validated in many biological systems [14-16], it is unclear how well the evolutionary trajectories of human opportunistic pathogens can be predicted by this model. A fundamental problem in applying the trade-off hypothesis to opportunists is that it assumes the pathogen relies strictly on its host for survival and host mortality is costly for the pathogen [11, 17]. However, many opportunistic pathogens are non-obligate and capable of living independently of their host [13, 17, 18]. In fact, it has been proposed that higher environmental persistence of a pathogen may lead to increased virulence in natural systems by relaxing the transmission-virulence constraint that otherwise confines obligate pathogens [19, 20].

Here, we tested the evolution of virulence of an opportunistic pathogen in a chronic infection, using the human opportunist *P. aeruginosa* in a murine chronic wound model. *P. aeruginosa* is an ESKAPE pathogen notorious for multi-drug resistance [21], and a model organism for the study of chronic infections. It causes human infection in the lungs of cystic fibrosis (CF) patients, chronic wounds, and burn wounds [22]. *P. aeruginosa* is one of the most common bacterial pathogens isolated from chronic wounds, often forming antimicrobial-resistant biofilms that are difficult to eradicate [23]. Chronic wounds present a massive burden on individuals and healthcare systems worldwide [24-31]. They are characterized by persistent infection, excessive inflammation, and a significantly delayed healing process, and as a result, can be challenging and costly to treat [27].

While the adaptation of *P. aeruginosa* to CF lungs has been well-studied [32-35], long-term adaptation in chronic wounds is not as well-documented, presenting an opportunity to explore the nature of pathogenesis in a clinically relevant system. Using a two-part serial passage selection and sepsis experiment, and by relieving the pressure of transmission potential and maintaining evolution within the host, we tested whether virulence would evolve to increasing levels, as would be predicted by the trade-off hypothesis. To additionally further our understanding of *P. aeruginosa* adaptation in a chronic wound, we assessed phenotypic heterogeneity after ten rounds of selection, and used next generation sequencing to identify potential genetic signatures of *P. aeruginosa* adaptation to chronic wounds.

RESULTS

Wound bed and spleen bacterial population densities are positively correlated. To assess the adaptation trajectories of *P. aeruginosa* in a chronic wound, we used a serial passage selection experiment **(Fig. 1A),** where we established three independent evolution lines (A, B, and C) by infecting three mice with ~10^3 cells of the *P. aeruginosa* strain PA14 from a liquid culture. Each infection duration was 72 hours, after which we scarified the mice and harvested their wound bed and spleen tissues for colony forming unit (CFU) counts. We used a 1:1000 serial dilution of the wound bed infection to start a new liquid culture and inoculate the next mouse in each line of evolution, again with ~10^3 cells. We carried this selection experiment through a total of 10 rounds of mice for each of the three parallel evolution lines (n=30 mice in total). We assessed the changes in bacterial load during the course of selection and found that wound bed CFUs throughout the serial passage experiment were fairly constant within two orders of magnitude, aside from one mouse in evolutionary line A at the 8th round, whose CFUs were notably lower (**Fig. 2A**). The number of bacterial cells derived from spleens were highly variable across all three lines of evolution, with many values being below the limit of detection, 10^2 cells (**Fig. 2B**). There was a positive correlation between wound bed and spleen CFUs during the serial passage experiment, $r(28) = .44$, $p = .015$.

Phenotypic heterogeneity is limited in chronic wound adapted populations. As phenotypic diversity has been extensively reported in cystic fibrosis (CF) infections of *P. aeruginosa* [32-34, 36, 37], we were interested in characterizing *P. aeruginosa* adaptation to a chronic wound and assessing population heterogeneity after ten rounds of selection. We began by characterizing the morphology of 100 isolates from populations of the 5th and 10th rounds of each evolutionary line. We initially assessed the possible changes in biofilm colony morphology types (morphotypes) using Congo red agar plates. At the 5th round of selection, each evolutionary line had only 1-2 distinguishable morphotypes. At the 10th round, line A had two distinguishable morphotypes, while lines B and C each had three (**Fig. 3A**).

We chose one representative isolate from each morphotype and line of evolution for further phenotypic analysis. Isolates are named for their evolutionary line and the order in which they were characterized, e.g. isolate A1 was the first isolate characterized in line A. We selected the following isolates for further testing: A88, A92, B16, B31, B42, C31, C38, and C62 (**Table 1; Fig. 3A**). We tested the isolates for total protease production, pyoverdine, pyochelin, pyocyanin, swimming and swarming motilities, as these functions play an active role in *P. aeruginosa* pathogenesis [22]. Isolates A92, B16, C38, and C62 had similar levels of protease production to

PA14; while A88, B31, B42, and C31 had decreased relative protease activity (**Fig. 3B**). A88, A92, B16, B31, and C31 had intact swimming motility; however, only A92 and B16 had functioning swarming motility (**Fig. 3B-C**). There were no appreciable differences in pyoverdine ($\chi^2(8) = 13.347$, $p = .1005$), pyochelin ($\chi^2(8) = 13.754$, $p = .0884$), or pyocyanin ($\chi^2(8) = 14.551$, $p = .06848$) production amongst isolates (**Fig. 4**).

Genes encoding virulence factors are mutated during evolution in a chronic wound. We next conducted next-generation whole genome sequencing on each of the representative morphotypes to identify possible genetic signatures of adaptation to a chronic wound. A number of mutations were identified, many occurring in more than one isolate and across more than one line of evolution. Across all three lines, we found in total eight unique mutations, six of them resulting in a change in amino acid sequence (**Table 2**). From the list of all mutations, we chose to focus on the latter six mutations.

Two isolates, A92 and B16, had no non-synonymous mutations differing from the ancestor. At least one isolate in each line (A88, B31, B42, and C31) contained a mutation in both *lasR* and *pvcA*. Two isolates from line C (C38 and C62) were *fleQ* mutants, and also had frameshift mutations in a gene encoding a hypothetical protein. B42 was the only *rpoN* mutant, while C62 was the only *pilR* mutant in all three lines. *lasR*, *pvcA*, *fleQ*, *rpoN*, and *pilR* are all genes implicated in *P. aeruginosa* virulence [38-49], highlighting that genes encoding traditional virulence factors acquired mutations during adaptation to a chronic wound environment.

Virulence can divergently evolve in a chronic wound. Finally, we compared the virulence of each of the three evolved populations against each other and the ancestor PA14 using a sepsis experiment (**Fig. 1B**). We began the sepsis experiment by growing liquid cultures of the three final evolved populations and the ancestor PA14. We used each of these four liquid cultures to inoculate a distinct set of five mice with ~10^5 cells (n=20 mice in total). We monitored these mice for 80 hours for sepsis. If a mouse was moribund, it was sacrificed, time of death noted, and spleen harvested for CFU counts. At the end of 80 hours, all remaining mice were sacrificed and their spleens harvested for CFU counts. Because of the spleen's role in the host immune response and blood filtration during infection, it is often one of the first organs to become infected post-septicemia. As such, bacterial load in the spleen is a better indicator of systemic infection and more relevant when discussing virulence and host health, while wound bed bacterial load is primarily an indicator of infection severity at the site of infection. Therefore, we chose to only measure spleen colony forming units (CFUs) for the sepsis experiment.

We observed that at the end of the sepsis experiment, three of the five mice infected by evolution line C had survived, two from the ancestor PA14, one from line A, and none from line B (**Fig. 5A**). A Kruskal-Wallis test showed that there were significant differences in the mean spleen CFUs at time of death between mice infected by the various populations ($\chi^2(3) = 10.623$, $p = .014$; **Fig. 5B**). A *post hoc* analysis showed that this statistically significant difference was between mice infected by lines B and C ($p = .023$, Dunn's test, Holm-Bonferroni correction). Mice infected by the ancestor PA14 and line B showed some level of difference in spleen CFUs, just above the $\alpha = .05$ significance threshold ($p = .058$). From this data we found that over the course of ten rounds of selection, line B evolved to be more virulent, line C evolved to be slightly less virulent, and line A remained approximately as virulent as the ancestor.

DISCUSSION

Opportunistic pathogens and their resulting infections are a significant human healthcare concern, but by understanding their pathogenesis, it is possible to gain insights for improved clinical treatments and novel therapies. To assess how opportunists evolve virulence in chronic infections, we passaged *P. aeruginosa* PA14 for ten rounds of selection in a mouse chronic wound model over the course of 30 days, then compared the virulence of the evolved populations with the ancestor using a mouse sepsis experiment. Based upon the fundamental assumptions of the trade-off hypothesis, if a pathogen's constraint of transmission potential is relieved, virulence is predicted to increase, as host survival will no longer determine which pathogen populations are able to transmit to new hosts [6, 50]. Our findings were not consistent with this prediction; in fact, we saw that virulence evolved differently in each line. No single virulence model on its own is able to adequately explain this diverging pattern, suggesting there may be previously overlooked variables influencing virulence evolution in opportunistic infections, or that components from multiple models may need to be considered in tandem.

The role of multiple infection may partially account for what we observed, as within-host adaptation leading to infection by multiple genotypes can result in higher or lower virulence, depending on the context. Levin & Bull originally proposed the short-sighted evolution hypothesis to explain the role of multiple infection and within-host selection on virulence [51]. According to their model, as a strain mutates and diversifies within the host, competition for limited resources will favor fast-growing genotypes, leading to higher virulence. Therefore, pathogens are short-sighted in that they respond to immediate within-host selection pressures and sacrifice their long-term evolutionary advantage by harming the host. This is in accordance with the result anticipated by relaxing the transmission constraint of the trade-off hypothesis.

For non-obligate environmental persisters such as *P. aeruginosa*, increasing virulence would seemingly be the optimum. Why then, did we observe lowered virulence in one of the selection lines? For social bacterial pathogens like *P. aeruginosa*, there is a strong link between social traits and virulence, as the production of many virulence factors is controlled by quorum sensing (QS). When social pathogens undergo within-host diversification, they are susceptible to the rise of QS mutants that undermine the pathogenicity of the entire population. These mutants or 'cheats' do not pay the cost of producing social goods, yet are still able to reap the benefits of those produced by cooperators [7, 52-54]. It has been shown that, through the principle of kin selection, *P. aeruginosa* populations with high genetic relatedness in regards to QS genes tend to select for cooperative QS, and as a result, higher virulence, while those with low genetic relatedness tend to favor cheats, resulting in lower virulence [55, 56]. In future work, exploring genetic diversity using deep sequencing may provide insight as to whether the role of coordinated social behaviors and QS mutant frequency can explain the variation in virulence between our evolved populations.

There are a number of other factors to consider in order to make meaningful predictions for *P. aeruginosa* virulence in human infections: (i) environmental selection pressures, (ii) host immune response, and (iii) mixed-species interactions [13, 18, 57-61]. As a non-obligate opportunist, *P. aeruginosa* faces unique selection pressures from both the external environment and within the host. The coincidental selection hypothesis speculates that in non-obligates, virulence may evolve

in the environment and only persist in the host due to a lack of selection pressure otherwise. Thus, the pathogen's history of transmission, and whether it was acquired environmentally or via human contact may impact the virulence of infection. Host immunity also plays a key role in infection dynamics and virulence evolution, as *P. aeruginosa* is known to change its expression of virulence factors directly in response to immune activation by the host [62], and has a number of means of immune evasion, such as loss of swimming motility, modifications to lipopolysaccharide (LPS) structure, and decreased expression of type III secretion system (T3SS) components [63, 64]. Variations in host immune response may drive the pathogen toward different defenses, leading to different outcomes for virulence. Lastly, while we chose to focus on *P. aeruginosa* and follow the diversification of one strain, chronic wounds are typically infected with a diverse suite of microbes, such as *Staphylococcus aureus*, *Enterococcus faecalis*, *Proteus* spp., *Escherichia coli*, and coagulase-negative staphylococci [23, 65]. Interspecies competition and co-existence may provide novel niches or selection pressures that influence virulence. Host immunity and mixed-species interactions cannot be ruled out as variables in our experiment, as they are not entirely standardized even in laboratory mice, who may have small genetic variations and unique flora. Furthermore, given all of these considerations, caution must be exercised when extrapolating experimental results from a simplified laboratory mouse model to a vastly more complex human infection.

We were also interested in characterizing *P. aeruginosa* adaptation to a chronic wound, and found that (i) there is a lower degree of phenotypic diversity in chronic wounds than is seen in other chronic infections of *P. aeruginosa*, and (ii) *P. aeruginosa* acquires mutations in major regulators and genes involved in pathogenesis during adaptation to a chronic wound. We did not observe extensive phenotypic diversity after ten rounds of selection in a chronic wound, such as is seen in CF lungs [34, 36, 37]. This may be due to the time scale of our experiment, as 30 days is not comparable to years of development in a CF lung, or that a chronic wound lacks the spatial structure that is seen in CF lungs, providing fewer niches for diversification. We must also consider that while our experiment focused on *P. aeruginosa*, CF lungs are comprised of polymicrobial infections, which may encourage further diversification through competition and cooperation.

In our chronic wound evolved populations, we discovered mutations in *lasR*, *pvcA*, *fleQ*, *rpoN*, and *pilR*, genes that are implicated in *P. aeruginosa* virulence via QS, elastase, iron chelation, motility, type IV pili, surface attachment, and biofilm formation [38-49]. Many of these mutations correlate with the phenotypes we observed, such as decreased protease production in *lasR* mutants, and inhibited motility in *fleQ* and *rpoN* mutants. Although *lasR* is not directly involved in swarming motility, swarming is a complex behavior that requires cell-to-cell signaling, and it has been previously shown that *lasR* mutants may show diminished swarming behavior [66]. Likewise, we saw in our experiment that, in addition to the *rpoN* and *fleQ* mutants, *lasR* mutants were also unable to swarm. It has previously been shown that in chronic CF infections, *P. aeruginosa* selects against the production of virulence factors that are required for acute infection [34, 67]. This may be a means of host immune evasion, or because virulence factors are costly to produce and no longer necessary beyond infection colonization [67]. Namely, many CF isolates are *lasR*, *rpoN*, and *fleQ* mutants [32-35]. Our results, taken with previous studies on CF isolates, suggest that *P. aeruginosa* may employ similar adaptations in a chronic wound.

Overall, our findings emphasize that there is still much work to be done towards understanding the dynamics and drivers of virulence evolution of opportunistic pathogens in chronic infections. We

have shown that, contrary to the trade-off hypothesis, virulence does not necessarily increase even when the restriction of transmission potential is removed. In fact, evolution was not reproducible in the three independent selection lines, in contrast to a previous study that showed *P. aeruginosa* evolution was highly reproducible *in vitro* [68], further highlighting the importance of factoring host-specific variables such as immune response in predictions of virulence. Our study also adds to the breadth of knowledge on *P. aeruginosa* adaptations in chronic wounds, showing that while *P. aeruginosa* employs similar adaptive strategies, i.e. loss of virulence factors, in both chronic wounds and CF lungs, diversification in chronic wounds is far less extensive. With our work and suggestions for future studies, we hope to provide insights for improved chronic infection virulence management strategies.

MATERIALS & METHODS

Bacterial Strains and Culture Conditions. We inoculated mice with the *Pseudomonas aeruginosa* strain PA14. For overnight cultures, we grew in 24-well microtiter plates in lysogeny broth (LB) and incubated at 37°C with shaking at 200 RPM.

Serial passage experiment. The murine chronic wound model used in this study is based on one that has been previously described [69-74]. We anesthetized Female Swiss Webster mice (Charles River Laboratories, Inc.), weighing an average of 25g, by intraperitoneal injection of 100 mg/kg sodium pentobarbital (Nembutal; Diamondback Drugs). Subsequently, we administered a dorsal 1.5 x 1.5 cm excisional skin wound to the level of the panniculus muscle. We then covered wounds with transparent, semipermeable polyurethane dressings (OPSITE dressings) and injected approximately 10^3 bacterial cells in LB into the wound bed to establish infection. This adhesive dressing prevents contractile healing and ensures that these wounds heal by deposition of granulation tissue, much like human wounds. At the end of the experimental infection period, we sacrificed the animals and harvested their wounds and spleens for colony forming unit (CFU) quantification on Pseudomonas Isolation Agar (PIA). We collected and saved a lawn of the 1:1000 dilution of each population in BHI + 25% glycerol. For each mouse, we re-grew the cryo-stored wound population of the previous mouse in a new LB culture and inoculated, as before, into a new animal. Three parallel groups of 10 mice were used (n=30), with the initial mouse of each group being inoculated with a stock population of PA14.

Sepsis model. From each of the 10th and final mouse populations from the serial passage experiment, along with the ancestor PA14, we grew the cryo-stored wound population in LB and injected 10^5 bacterial cells into the wound bed of five new mice (n=20). We monitored these mice for 80 hours for sepsis. If a mouse was moribund during this period, we sacrificed it and harvested its spleen for CFU counts. At the end of 80 hours, we sacrificed all remaining mice and harvested spleens for CFU counts.

Whole genome sequencing. We plated serial dilutions of the previously cryo-stored populations on Congo Red agar (CRA) plates. We randomly picked 100 colonies from each population of interest to start overnight cultures, and from these, made cryo-stocks of each isolate and plated on CRA to compare colony morphologies. We streaked out the cryo-stocks on LB agar and picked single colonies, from which we grew overnight cultures in lysogeny broth. We isolated genomic DNA from the liquid cultures using the DNeasy Blood and Tissue Kit (Qiagen) according to the

manufacturer's instructions. We prepared sequencing libraries using the Nextera XT DNA Library Preparation Kit and sequenced with the Illumina Miseq platform, with a minimum average calculated level of coverage of 30X for each selected isolate. We analyzed the sequencing data using the reference-based alignment and variant calling tool *breseq* with default parameters.

Pyocyanin assay. Our pyocyanin assay is based on one that has been previously described [75]. We grew all isolates overnight in LB, then standardized OD_{600} of all cultures using phosphate-buffered saline (PBS). We spun cultures down briefly in a microcentrifuge before filtering through 0.2µm pore size syringe filters. We extracted 1mL of filter sterilized supernatant with 600µL chloroform, vortexed for 2 minutes, then centrifuged at 10,000 rpm for 5 minutes. We re-extracted the resulting blue layer, discarding the colorless layer, with 400µL of 0.2M HCl, vortexed again for 2 minutes, and centrifuged at 10,000 rpm for 5 minutes. We then transferred the pink layer into a 96-well plate (Corning) and read the optical density at 520 nm.

Pyoverdine and pyochelin assay. Succinate media and siderophore assay are modified from multiple sources [76-81]. Succinate media used for these assays is composed of 6g K_2HPO_4, 3g KH_2PO_4, 1g $(NH_4)_2PO_4$, 0.2g $MgSO_4$, and 4g succinic acid in 1L H_2O, pH adjusted to 7. We first grew all isolates overnight in LB, spun down 500µL of overnight LB culture, rinsed 2X with equal volume succinate media, and used this starter culture to inoculate 5mL of succinate media. We grew succinate cultures for 36 hours at 30°C. We standardized OD_{600} of all cultures using PBS, filtered cultures using 0.2µm pore size syringe filters, and transferred 100µL of supernatant to a black 96-well microtiter plate. We measured pyoverdine fluorescence with an excitation wavelength of 400nm, emission wavelength of 460nm, and gain of 61. We measured pyochelin fluorescence with an excitation wavelength of 350nm, emission wavelength of 430nm, and gain of 82.

Protease activity. Skim milk agar is composed of 5% w/v dry milk with 1.25% w/v agar. We poured 15mL of skim milk agar in 100 x 15mm Petri dishes. We grew liquid cultures overnight from a single colony in LB, then standardized OD_{600} of all cultures using PBS. We spun cultures down briefly in a microcentrifuge before filtering through 0.2µm pore size syringe filters. We spotted 10µL of filtered supernatant on skim milk agar plates, using 10µL of LB as a negative control and 1µL of proteinase K as a positive control. We incubated plates at 37°C overnight and measured the zone of protease activity qualitatively.

Swarming motility. The recipe for swarm agar and experimental protocol are adapted from multiple sources [66, 82, 83]. Swarm agar is composed of 1X M8 salt solution, 0.6% w/v agar, 0.5% w/v casamino acid, 0.2% w/v glucose, and 1mM $MgSO_4$. We poured 25mL of swarm agar in 100 x 15mm Petri dishes under laminar flow, allowing for plates to dry for 30 minutes with lids off. We grew liquid cultures overnight from a single colony and inoculated plates with 2.5µL of overnight culture, incubating in short stacks of ≤4 plates, right side up for approximately 20 hours.

Swimming motility. Swim agar is composed of LB with 0.3% agar. We poured 25mL of swim agar in 100 x 15mm Petri dishes, allowing a few hours to dry at room temperature with lids closed. We grew isolates overnight from a single colony, dipped a toothpick into the overnight culture, and inoculated by sticking the toothpick in the center of each plate, halfway through the agar. We

wrapped short stacks of ≤4 plates in cellophane and incubated overnight for 20 hours at 37°C alongside two large containers of water to retain humidity in the incubation chamber.

Statistical analysis. We used a Kruskal-Wallis one way test of variance to test for the difference of means, followed by a *post hoc* Dunn's test with a Holm-Bonferroni family-wide error rate (FWER) correction. We used a Pearson's correlation test to test the linear correlation between variables. Statistical significance was determined using a p-value < .05. The creation of graphs and statistical analyses were conducted in R version 3.6.1 using the packages tidyverse [84], ggplot2 [85], ggpubr [86], and PMCMR [87].

Ethical Statement. All animals were treated humanely and in accordance with protocol 07044 approved by the Institutional Animal Care and Use Committee at Texas Tech University Health Sciences Center in Lubbock, TX.

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FIGURE & TABLE LEGENDS

Figure 1. Serial passage and sepsis experimental protocols. A) Serial passage experimental protocol. We established three independent evolution lines by infecting three mice with ~10^3 cells of the *P. aeruginosa* strain PA14 from a liquid lysogeny broth (LB) culture. Each infection duration was 72 hours, after which we scarified the mice and harvested their wound bed and spleen tissues for colony forming unit (CFU) counts on *Pseudomonas* isolation agar (PIA). We used a 1:1000 serial dilution of the wound bed infection to start a new LB liquid culture and inoculate the next mouse in each line of evolution, again with ~10^3 cells. We carried this selection experiment through a total of 10 rounds of mice for each of the three parallel evolution lines (n=30 mice in total). One replicate evolutionary line is shown in the diagram. B) Sepsis experimental protocol. We began the sepsis experiment by growing LB liquid cultures of the three final evolved populations from the serial passage experiment and of the ancestor PA14. We used each of these four liquid cultures to inoculate a distinct set of five mice with ~10^5 cells (n=20 mice in total). We monitored these mice for 80 hours for sepsis. If a mouse was moribund, it was sacrificed, time of death noted, and spleen harvested for CFU counts. At the end of 80 hours, all remaining mice were sacrificed and their spleens harvested for CFU counts.

Figure 2. Changes in population density during serial passage experiment. A) Wound bed CFUs for mice at time of death for each evolutionary round were relatively stable, aside from the 8th mouse in line A. B) Spleen CFUs for mice at time of death for each evolutionary round were

highly variable throughout the experiment, with many values falling below the limit of detection, 10^2 cells. Wound bed and spleen CFUs during the serial passage experiment were positively correlated, $r(28) = .44$, $p = .015$.

Figure 3. Phenotypic heterogeneity is limited in evolved populations. A) There are eight distinct types of biofilm colony morphology on CRA at the final round of selection across all three lines of evolution, with line A having two distinct morphology types, and lines B and C each having three distinct colony morphology types. B) Isolates A92, B16, C38, and C62 have protease activity comparable to that of the ancestor PA14, while isolates A88, B31, B42, and C31 have decreased protease activity. C) Isolates B42, C38, and C62 have lost the ability to swim. D) Isolates A88, B31, B42, C31, C38, and C62 have lost the ability to swarm.

Figure 4. Heterogeneity in pyoverdine, pyochelin, and pyocyanin production is limited in evolved populations. A) Pyoverdine production in across final evolved representative isolates. Error bars indicate SEM. B) Pyochelin production in across final evolved representative isolates. C) Pyocyanin production in across final evolved representative isolates.

Figure 5. Virulence can evolve in diverging directions in a chronic wound. A) Mice infected by the final evolved population of line B in the sepsis experiment had the highest mortality rate (100%), with no surviving mice at the end of 80 hours, while mice infected by line C had the lowest mortality (40%), with three of five mice surviving. B) Mice infected by the final evolved population of line B in the sepsis experiment had significantly higher mean spleen CFUs at time of death as compared to mice infected by line C, indicating more severe septicemia (Kruskal-Wallis, Dunn's *post hoc* test, Holm-Bonferroni correction, $p = .023$). Error bars indicate SEM.

Table 1. Details of the eight distinct biofilm colony morphology types on CRA of evolved isolates after ten rounds of selection across all three lines of evolution. One isolate of each distinct colony morphology type from each population was selected as a representative for further phenotypic assays and sequencing analysis.

Table 2. A list of all mutations in the final evolved representative morphology type isolates as mapped to the PA14 reference genome, including synonymous mutations. Many genes coding for virulence factors or regulators of virulence are mutated over the course of adaptation to a chronic wound.

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Figure 1

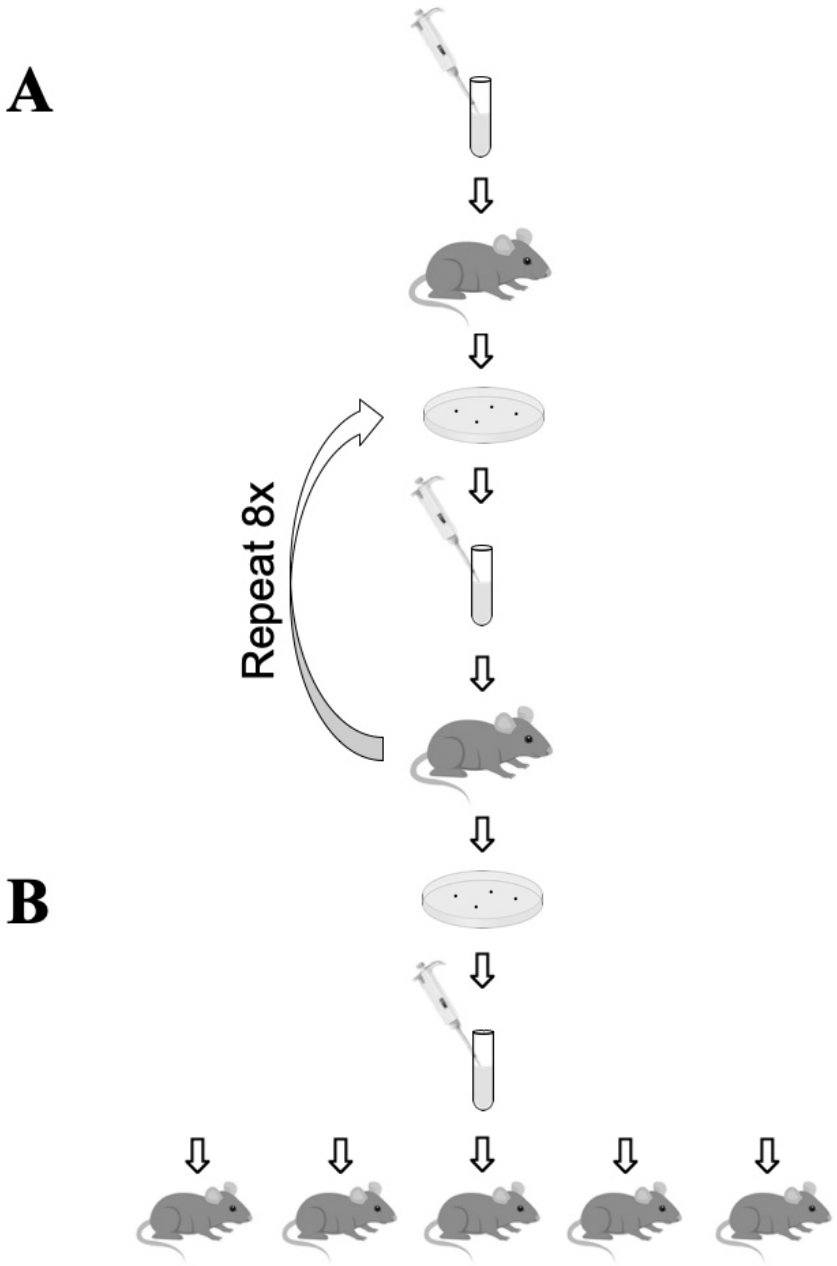

Figure 2

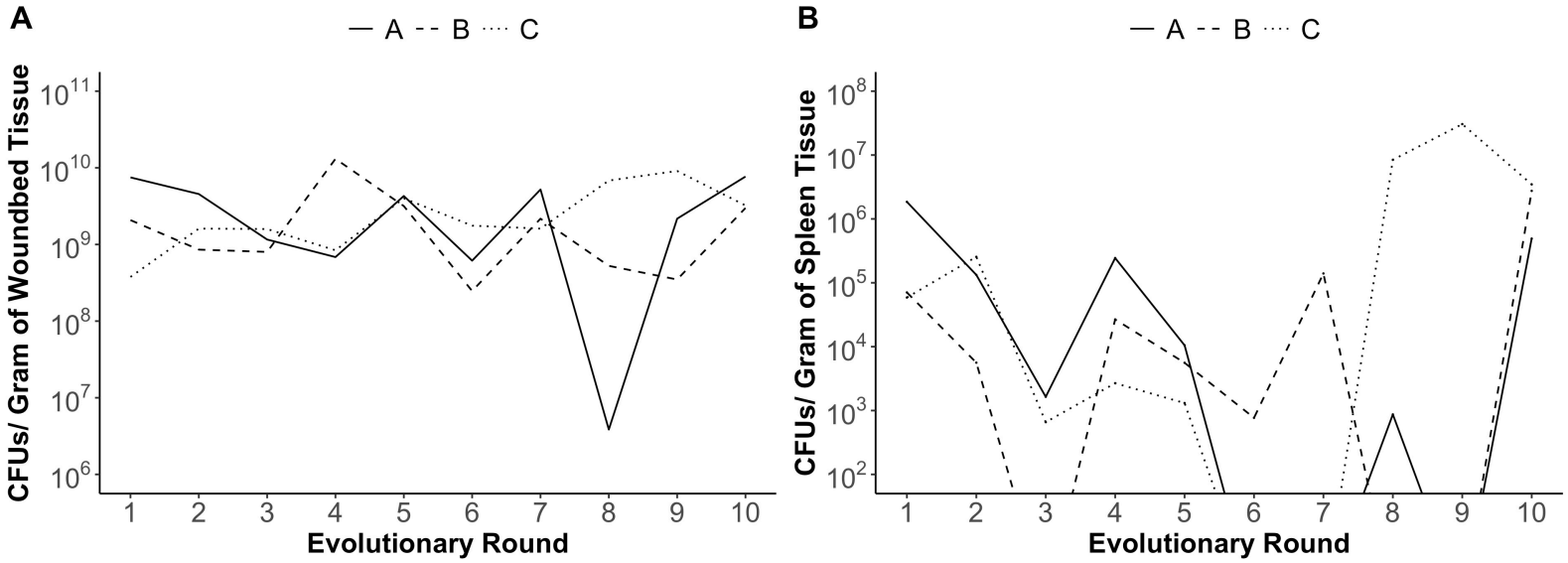
A
A B C
CFUs/ Gram of Woundbed Tissue
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
1 2 3 4 5 6 7 8 9 10
Evolutionary Round
B
A B C
CFUs/ Gram of Spleen Tissue
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
1 2 3 4 5 6 7 8 9 10
Evolutionary Round

Figure 3

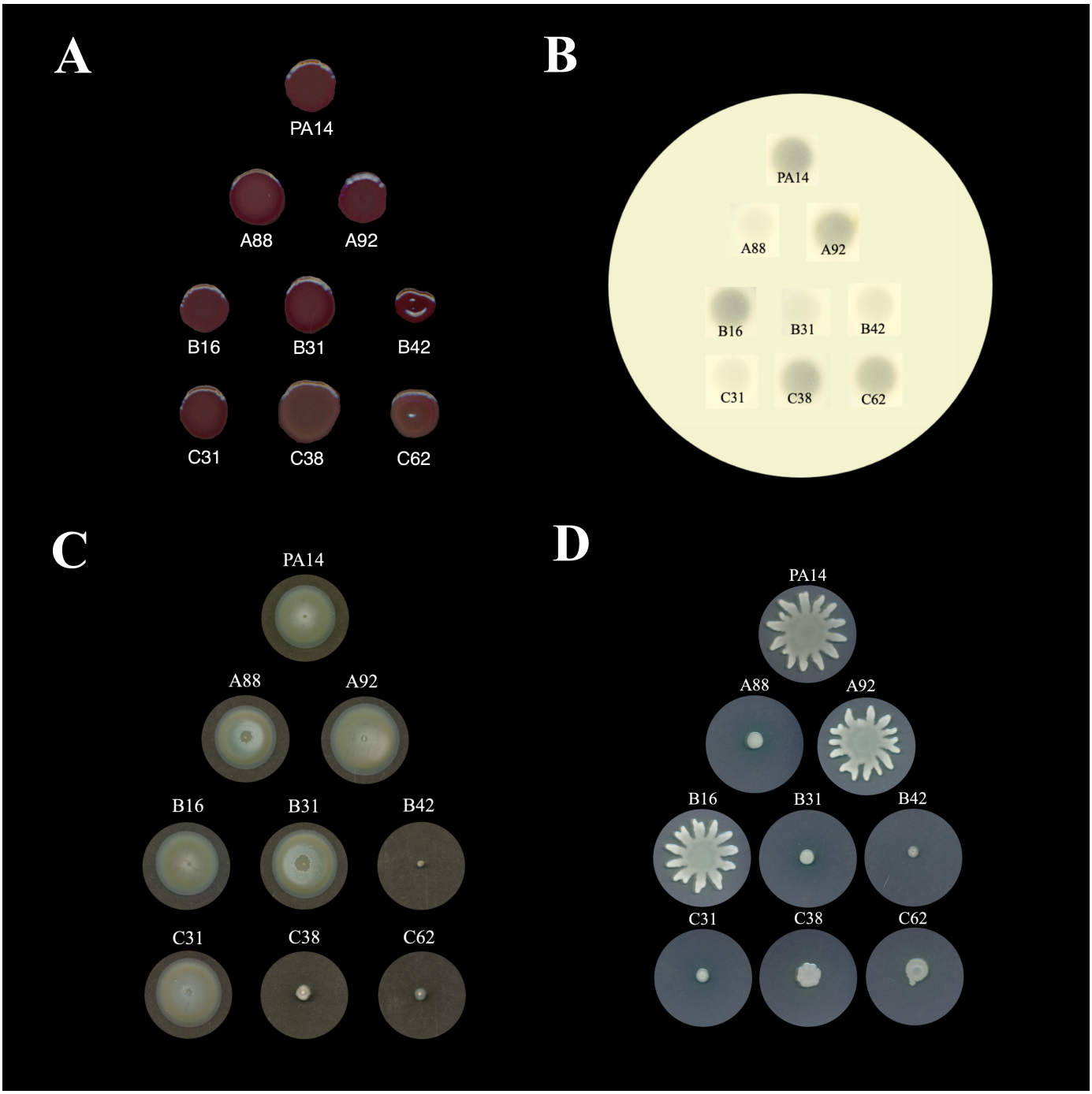
A
PA14
A88
A92
B16
B31
B42
C31
C38
C62
B
PA14
A88
A92
B16
B31
B42
C31
C38
C62
C
PA14
A88
A92
B16
B31
B42
C31
C38
C62
D
PA14
A88
A92
B16
B31
B42
C31
C38
C62

Figure 4

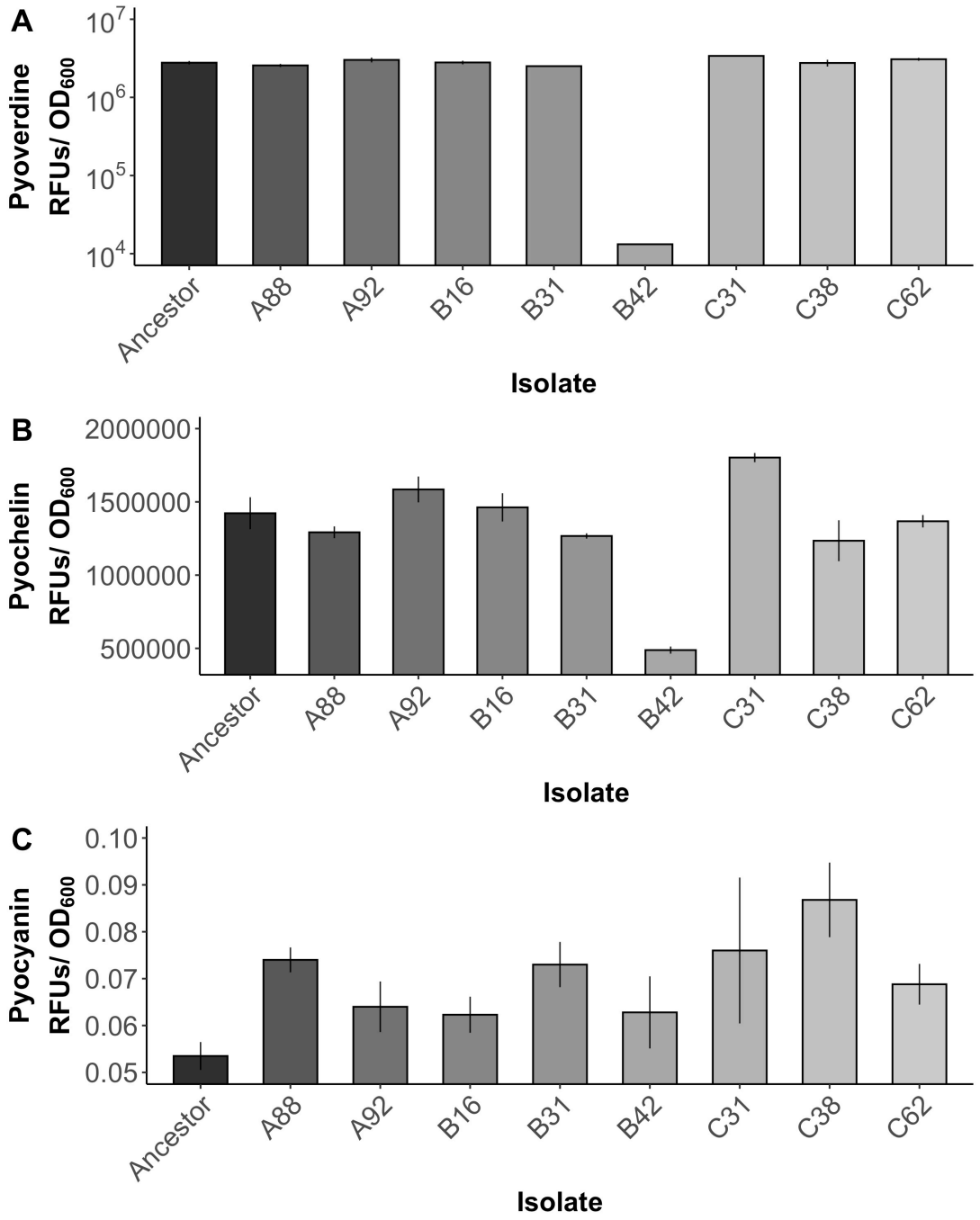
A
Pyoverdine
RFUs/ OD600
10^7
10^6
10^5
10^4
Ancestor
A88
A92
B16
B31
B42
C31
C38
C62
Isolate
B
Pyochelin
RFUs/ OD600
2000000
1500000
1000000
500000
Ancestor
A88
A92
B16
B31
B42
C31
C38
C62
Isolate
C
Pyocyanin
RFUs/ OD600
0.10
0.09
0.08
0.07
0.06
0.05
Ancestor
A88
A92
B16
B31
B42
C31
C38
C62
Isolate

Figure 5

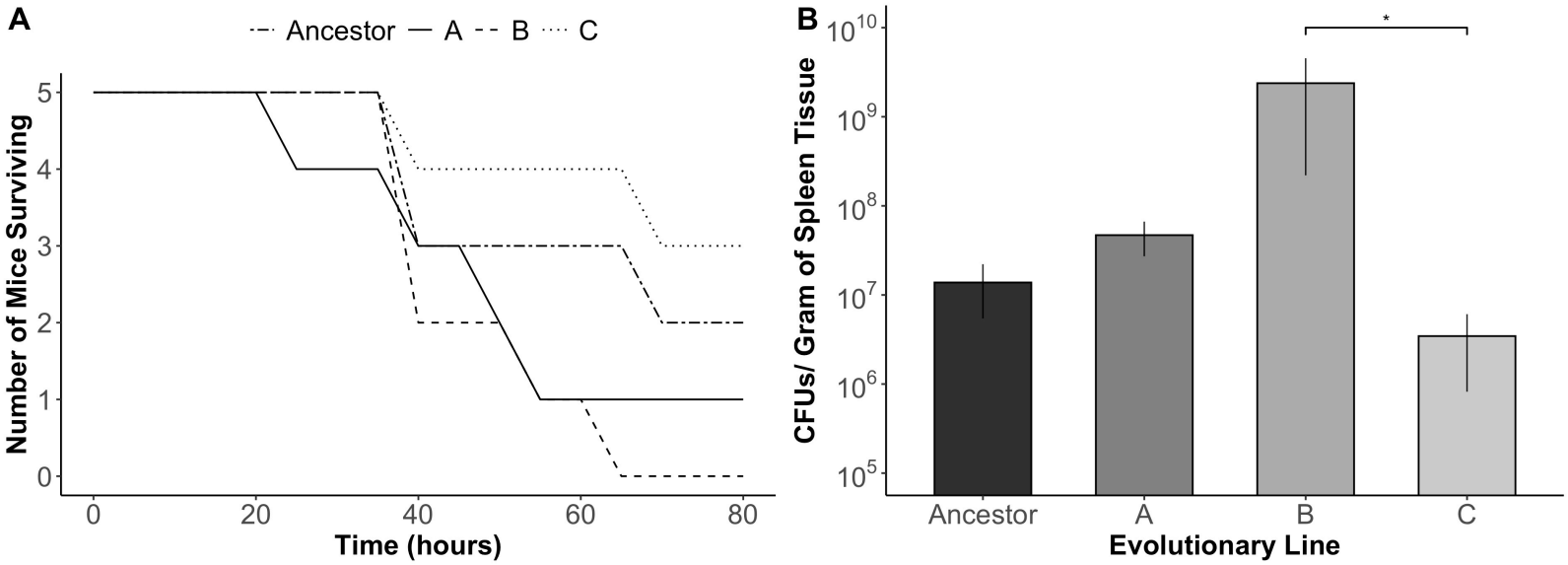
A
Ancestor
A
B
C
Number of Mice Surviving
0
1
2
3
4
5
0
20
40
60
80
Time (hours)
B
CFUs/ Gram of Spleen Tissue
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
*
Ancestor
A
B
C
Evolutionary Line

Table 1.

Representative Isolate	Morphology type	Morphology Description
PA14	1	dull red, wrinkly edge, smooth texture
A88	2	blood red, smooth edge, smooth texture
A92	1	dull red, wrinkly edge, smooth texture
B16	1	dull red, wrinkly edge, smooth texture
B31	2	blood red, smooth edge, smooth texture
B42	3	blood red, raised outer ring
C31	4	blood red, smooth edge, smooth texture
C38	1	dull red, wrinkly edge, smooth texture
C62	5	dull red, smooth edge, smooth texture, faint outer ring

Table 2.

Gene locus	Gene annotation	Genetic Mutation	Amino Acid Effect	Isolate(s)
PA14_33010	glyA2	TTC → TTT	F257F	A92, B16, B31, C31, C38
PA14_33290/ PA14_33300	intergenic region	Δ180 bp	N/A	C62
PA14_35430	*pvcA*	GCG → ACG	A249T	A88, B31, B42, C31
PA14_45960	*lasR*	Δ9 bp at pos. 130-138	ΔS44-D46	A88, B31, B42, C31
PA14_50220	*fleQ*	GTC → GGC	V270G	C38, C62
PA14_57940	*rpoN*	GAC → AAC	D459N	B42
PA14_60260	*pilR*	ACC → CCC	T275P	C62
PA14_70360	hypothetical protein	Δ36 bp at pos. 98-133	Frameshift Mutation	C38, C62