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

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ABSTRACT

Opportunistic pathogens are associated with a number of chronic human infections, yet the evolution of virulence in these organisms during chronic infection remains poorly understood. Here, we tested the evolution of virulence in the human opportunistic pathogen *Pseudomonas aeruginosa* in a murine chronic wound model using a two-part serial passage and sepsis experiment, and found that virulence evolved in different directions in each line of evolution. We also assessed *P. aeruginosa* adaptation to a chronic wound after 42 days of evolution, and found that morphological diversity in our evolved populations was limited compared to that previously described in cystic fibrosis (CF) infections. Using whole genome sequencing, we found that genes previously implicated in *P. aeruginosa* pathogenesis (*lasR*, *pilR*, *fleQ*, *rpoN*, and *pvcA*), acquired mutations during the course of evolution in wounds, with some mutations evolving in parallel across all lines of evolution. Our findings highlight that (i) *P. aeruginosa* heterogeneity may be less extensive in chronic wounds than in CF lungs; (ii) genes involved in *P. aeruginosa* pathogenesis acquire mutations during chronic wound infection; (iii) similar genetic adaptations are employed by *P. aeruginosa* across multiple infection environments, and (iv) current models of virulence may not adequately explain the diverging evolutionary trajectories observed in an opportunistic pathogen during chronic wound infection.

INTRODUCTION

Opportunistic pathogens, those that only cause disease in hosts with compromised immune defenses, are responsible for several chronic, treatment-resistant infections in humans, 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 in both community and hospital environments, 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 remain two core questions for which the answers are unclear: (i) how does virulence typically evolve in opportunistic pathogens, and (ii) are these patterns of evolution predictable?

Currently, there are four classic hypotheses which explain how pathogenic virulence evolves, where virulence is attributed to either (i) new host-parasite associations; (ii) short-sighted evolution; (iii) evolutionary trade-offs; or (iv) coincidental selection [5-7]. The first of these, the 'conventional wisdom' of early virulence evolution theory, speculates that pathogens should evolve over time towards avirulence or commensalism with the host, and that virulence is a reflection of a novel host-parasite association [8]. In contrast, the short-sighted evolution hypothesis postulates that pathogens evolve higher virulence in response to immediate within-host selection pressures, meanwhile sacrificing their long-term evolutionary advantage by harming the host [9]. The trade-off hypothesis predicts that pathogens will optimize their overall reproductive fitness by trading off between virulence and transmission, selecting for intermediate virulence [10]. Unlike the other models that look to within-host determinants, the coincidental selection hypothesis argues that virulence may be inconsequential to success in the host of interest, evolving in the environment or another host and merely maintained due to minimal impact on fitness [6]. While these models of virulence evolution have been studied extensively in a number of biological systems [10-12], there have been few empirical tests as to how virulence evolves in opportunistic pathogens during chronic infection [13].

Here, we tested how virulence evolves in an opportunistic pathogen during chronic infection, using the human opportunist, *P. aeruginosa,* in murine chronic wounds. *P. aeruginosa* is an ESKAPE pathogen notorious for multi-drug resistance [14] and a model organism for the study of chronic infections. It causes long-term infection in the lungs of cystic fibrosis (CF) patients and in chronic diabetic wounds [15]. *P. aeruginosa* is one of the most common bacterial pathogens isolated from chronic wounds, often forming antimicrobial-tolerant biofilms that are difficult to eradicate [16]. Chronic wounds present a massive burden on patients and healthcare systems worldwide, characterized by persistent infection, excessive inflammation, and a significantly delayed healing process [17-24]. While the adaptation of *P. aeruginosa* to CF lungs has been well-studied [25-28], long-term adaptation in chronic wounds is not as well-documented, presenting opportunities to study the nature of virulence evolution and pathogenesis in a clinically relevant environment.

Using a two-part serial passage selection and sepsis experiment, we determined how *P. aeruginosa* virulence evolves in murine chronic wounds, and whether the evolution of virulence was reproducible. We also ascertained morphological diversity and phenotypic changes after 42 days and ten rounds of evolution in wounds, and used whole genome sequencing to identify genetic signatures of *P. aeruginosa* adaptation to a chronic wound environment.

MATERIALS & METHODS

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

92 **Serial passage experiment**. The murine chronic wound model used in this study is based on one
93 that has been previously described [29-31]. We anesthetized adult female Swiss Webster mice
94 (Charles River Laboratories, Inc.), weighing between 20-25 g, by intraperitoneal injection of 100
95 mg/kg sodium pentobarbital (Nembutal; Diamondback Drugs), before their backs were shaved,
96 and the hair was cleanly removed with a depilatory agent. As a preemptive analgesic, 0.05 mL of
97 lidocaine (500 µL of bupivacaine [0.25% w/v] with 500 µL of lidocaine [2% w/v]) was injected
98 subcutaneously in the area to be wounded. We then administered a dorsal 1.5 x 1.5 cm excisional
99 skin wound to the level of the panniculus muscle, and covered it with transparent, semipermeable
100 polyurethane dressings (OPSITE dressings) and injected approximately 10^3 bacterial cells
101 suspended in LB into the wound bed to establish infection. This adhesive dressing prevents
102 contractile healing and ensures that these wounds heal by deposition of granulation tissue. At the
103 end of the 72 h experimental infection period, we euthanized the animals and harvested their
104 wound beds and spleens for colony forming unit (CFU) quantification on *Pseudomonas* Isolation
105 Agar (PIA). We collected and saved a lawn of the 1:1000 dilution of each wound bed population
106 in BHI + 25% v/v glycerol, then re-grew the cryo-stored population of the previous mouse in a
107 new LB culture and inoculated, as before, into a new animal (Fig. S1A). We used three parallel
108 groups of 10 mice (n=30 in total) to establish three independent evolution lines (A, B, and C), with
109 the initial mouse of each group being inoculated with a stock population of PA14.
110
111 **Sepsis model**. In a chronic wound model, sepsis is an important indicator of virulence, as
112 septicemia is one of the most life-threatening outcomes of a chronic wound in human patients.
113 From each of the 10th and final evolved populations from the serial passage experiment, along with
114 the ancestral PA14, we grew the cryo-stored wound populations in LB. We used each of these four
115 liquid cultures to inoculate a distinct set of five mice with ~10^5 bacterial cells (n=20 in total; Fig.
116 S1B). We monitored these mice for 80 hours for development of sepsis. If a mouse was moribund
117 during this period, we euthanized it and harvested the spleen for CFU counts. At the end of 80 h,
118 we euthanized all remaining mice and harvested spleens for CFU counts. Due to the spleen's role
119 in the host immune response and blood filtration during infection, it is often one of the first organs
120 to become infected post-septicemia. As such, bacterial load in the spleen is a better indicator of
121 systemic infection and more relevant when discussing virulence and host health, while wound bed
122 bacterial load is primarily an indicator of infection severity at the site of infection [32]. Therefore,
123 we chose to only measure spleen colony forming units (CFUs) for the sepsis experiment.
124
125 **Colony morphology.** To assess the diversity in colony morphology of evolved populations, we
126 plated serial dilutions of the previously cryo-stored populations of evolutionary rounds 5 and 10
127 on Congo red agar (CRA) plates [33]. We chose CRA to highlight any rare colony morphology
128 types that may otherwise be overlooked on LB agar. We randomly picked 100 colonies from each
129 of these populations to start overnight cultures, and from these, made cryo-stocks of each isolate
130 and plated 1µL on CRA to compare individual colony morphologies. All CRA plates were
131 incubated at 37°C overnight, then for 3-4 days at room temperature to allow for full development
132 of colony morphologies.
133
134 **Whole genome sequencing and variant calling.** We chose one representative isolate from each
135 morphotype and line of evolution at round 10 in addition to the ancestral PA14 for sequencing
136 analysis. We streaked the cryo-stocks of these isolates on LB agar and picked single colonies, from
137 which we grew overnight cultures in LB broth. We isolated genomic DNA from the liquid cultures

138 using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. We
139 prepared sequencing libraries using the Nextera XT DNA Library Preparation Kit and sequenced
140 with the Illumina Miseq platform, with a minimum average calculated level of coverage of 30×
141 for each selected isolate. We first trimmed reads and removed adapter sequences, then mapped all
142 samples against *P. aeruginosa* PA14 (RefSeq accession number GCF_000404265.1), and called
143 single nucleotide polymorphisms (SNPs), insertions, and deletions using the reference-based
144 alignment and variant calling tool *breseq* with default parameters. We manually parsed this list to
145 eliminate any mutations erroneously called due to errors in sequencing alignment. Lastly, we
146 determined the variants called between the reference PA14 and our ancestral PA14, then manually
147 checked these against the list of indels and SNPs of all evolved isolates to create the final table of
148 evolved mutations. We confirmed all mutations occurring in coding regions of defined proteins
149 with Sanger sequencing.
150
151 **Pyocyanin assay.** The pyocyanin assay is based on one that has been previously described [34].
152 We grew all isolates overnight in LB, then standardized OD_{600} of all cultures to 1.0 using
153 phosphate-buffered saline (PBS). We spun cultures down briefly in a microcentrifuge before
154 filtering through 0.2µm pore size syringe filters. We extracted 1mL of filter sterilized supernatant
155 with 600µL chloroform, vortexed for 2 minutes, then centrifuged at 10,000 rpm for 5 minutes. We
156 discarded the clear layer and re-extracted the blue layer with 400µL of 0.2M HCl, vortexed again
157 for 2 minutes, and centrifuged at 10,000 rpm for 5 minutes. We then transferred the pink layer into
158 a clear 96-well plate (Corning) and read the optical density at 520 nm.
159
160 **Pyoverdine and pyochelin production.** Succinate media and siderophore production assay were
161 modified from multiple sources [35-40]. Succinate media used for these assays was composed of
162 6g K_2HPO_4, 3g KH_2PO_4, 1g $(NH_4)_2PO_4$, 0.2g $MgSO_4$, and 4g succinic acid to a final volume of
163 1L H_2O, pH adjusted to 7. We first grew all isolates overnight in LB, spun down 500µL of
164 overnight LB culture, rinsed 2× with equal volume succinate media, and used this starter culture
165 to inoculate 5mL of succinate media. We grew succinate cultures for 36 h at 30°C, as this medium
166 and culture condition has been shown to maximize siderophore production [39], so as to highlight
167 differences in production capability between isolates. We filtered cultures using 0.2µm pore size
168 syringe filters, and transferred 100µL of supernatant to a black 96-well clear bottom microtiter
169 plate (Corning). We measured pyoverdine fluorescence with an excitation wavelength of 400nm,
170 emission wavelength of 460nm, and gain of 61. We measured pyochelin fluorescence with an
171 excitation wavelength of 350nm, emission wavelength of 430nm, and gain of 82. We standardized
172 all fluorescence values by the OD_{600} of each culture.
173
174 **Protease activity**. We prepared skim milk agar plates composed of 5% w/v dry milk with 1.25%
175 w/v agar. We poured 15mL of skim milk agar in 100 × 15mm Petri dishes. We grew liquid cultures
176 overnight from a single colony in LB, then standardized OD_{600} of all cultures to 1.0 using PBS.
177 We spun cultures down briefly in a microcentrifuge before filtering through 0.2µm pore size
178 syringe filters. We spotted 10µL of filtered supernatant on skim milk agar plates, using 10µL of
179 LB as a negative control and 1µL of proteinase K as a positive control. We incubated plates at
180 37°C overnight and measured the zone of protease activity qualitatively.
181
182 **Swarming motility**. The components for swarm agar and experimental protocol were adapted
183 from multiple sources [41-43]. Swarm agar was composed of 1× M8 salt solution (64g Na_2HPO_4

184 · $7H_2O$ or 30g Na_2HPO_4, 15g KH_2PO_4, and 2.5g NaCl to a final volume of 1L H_2O), 0.6% w/v
185 agar, 0.5% w/v casamino acid, 0.2% w/v glucose, and 1mM $MgSO_4$. We poured 25mL of swarm
186 agar in 100 × 15mm Petri dishes under laminar flow, allowing for plates to dry for 30 minutes with
187 plate lids off. We grew liquid cultures overnight from a single colony and inoculated plates with
188 2.5µL of overnight culture, incubating in short stacks of ≤4 plates, right side up for approximately
189 20 h.
190
191 **Swimming motility**. Swim agar was composed of LB with 0.3% w/v agar. We poured 25mL of
192 swim agar in 100 × 15mm Petri dishes, allowing a few hours to dry at room temperature with plate
193 lids closed. We grew isolates overnight from a single colony, dipped a toothpick into the overnight
194 culture, and inoculated by sticking the toothpick in the center of each plate, halfway through the
195 agar. We wrapped short stacks of ≤4 plates in cellophane and incubated overnight for 20 h at 37°C
196 alongside two large containers of water to retain humidity in the incubation chamber.
197
198 **Statistical analysis**. We used a Kruskal-Wallis one -way test of variance to test for the difference
199 of means, followed by a *post hoc* Dunn's test with either a Holm-Bonferroni family-wide error
200 rate (FWER) or Benjamini & Hochberg false discovery rate (FDR) correction. We used a
201 Pearson's correlation test to test the linear correlation between variables. Statistical significance
202 was determined using a p-value < 0.05. We plotted graphs and performed statistical analysis in R
203 version 3.6.1 using the packages tidyverse [44], ggplot2 [45], ggpubr [46], and PMCMR [47].
204
205 **RESULTS**
206
207 **Wound bed and spleen bacterial population densities are positively correlated**. We assessed
208 the changes in bacterial load during the course of selection and found that wound bed CFUs
209 throughout the serial passage experiment were generally within two orders of magnitude, aside
210 from one mouse in evolutionary line A at round 8, whose bacterial load was notably lower (Fig.
211 1A). The bacterial load in spleens was highly variable across all three replicate lines of evolution,
212 with many values being below our limit of detection, as the lowest serial dilution we plated was
213 10^{-2} (Fig. 1B). There was a positive correlation between bacterial load in wound bed and spleen
214 during the serial passage experiment (Pearson's $r(28) = .44$; $p = 0.015$).
215
216 **Morphological diversity is limited in chronic wound adapted populations.** As diversity has
217 been extensively reported in cystic fibrosis (CF) infections of *P. aeruginosa* [25-27, 48-50], we
218 therefore characterized *P. aeruginosa* adaptation to chronic wounds and assessed population
219 heterogeneity after 42 days of evolution. We began by characterizing the morphology of 100
220 random isolates from populations of rounds 5 and 10 of each evolutionary line. We assessed the
221 diversity in colony morphology types (morphotypes) using Congo red agar plates. At round 5, each
222 evolutionary line contained only 1-2 distinguishable morphotypes. At round 10, line A had two
223 distinguishable morphotypes, while lines B and C each had three (Fig. 2A; Table S1). Isolates are
224 named for their evolutionary line and the order in which they were characterized. We chose one
225 representative isolate from each morphotype and line of evolution (A88, A92, B16, B31, B42,
226 C31, C38 and C62) for further analysis.
227
228 We tested these representative isolates for total protease activity, pyoverdine, pyochelin, and
229 pyocyanin production, and swimming and swarming motility, to assess any phenotypic variation

potentially relevant to *P. aeruginosa* pathogenesis between the evolved isolates and the ancestor [15]. Isolates A92, B16, C38, and C62 produced similar levels of protease production to PA14; while A88, B31, B42, and C31 demonstrated decreased relative protease activity (Fig. 2B). A88, A92, B16, B31, and C31 displayed swimming motility; however, only A92 and B16 showed fully functioning swarming motility (Fig. 2B-C). There were differences in pyoverdine ($\chi^2(8) = 20.186$, $p = 0.0097$), pyochelin ($\chi^2(8) = 19.17$, $p = 0.014$), and pyocyanin ($\chi^2(8) = 47.843$, $p = 1.059e\text{-}7$) production between isolates (Fig. 3).

Genes encoding virulence determinants are mutated during evolution in a chronic wound. Loss of virulence factors and social traits through genetic mutations is commonly observed in *P. aeruginosa* isolates collected from chronic CF infections [51-53]; however, the genetic adaptations of *P. aeruginosa* to chronic wounds are less well described. We conducted whole genome sequencing on each of the representative morphotypes to identify possible genetic signatures of adaptation to chronic wounds. Only a small number of mutations were identified, some occurring across more than one line of evolution. Across all three lines, we found in total seven unique mutations, six of them resulting in a change in amino acid sequence within a coding region (Table 1). We identified mutations in *lasR*, *pvcA*, *fleQ*, *rpoN*, *pilR*, all genes previously implicated in *P. aeruginosa* virulence [54-65]. The same *lasR* and *pvcA* mutations were found in each of the three evolutionary lines. There was additionally one frameshift mutation located within a coding region for a hypothetical protein with no known homologs.

Virulence can evolve divergently in chronic wounds. To assess how virulence evolved in our wound model, we compared the virulence of each of the three evolved populations against each other and the ancestral PA14 using a sepsis experiment (Fig. S1B). We observed that at the end of the sepsis experiment, three of the five mice infected by evolution line C survived, two from the ancestral PA14, one from line A, and none from line B (Fig. 4A). A Kruskal-Wallis test showed that there were significant differences in the mean spleen CFUs at the time of death between mice infected by the various populations ($\chi^2(3) = 10.623$, $p = 0.014$; Fig. 4B). A *post hoc* analysis showed that this statistically significant difference was between mice infected by lines B and C ($p = 0.023$, Dunn's test, Holm-Bonferroni correction). Mice infected by the ancestral PA14 and line B showed differences in spleen CFUs, just above the $\alpha = 0.05$ significance threshold ($p = 0.058$). Overall, we found that over the course of 42 days, 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 chronic infections pose a significant healthcare burden, affecting 1-2% of the population in developed nations and amounting to billions of dollars annually in treatment costs [17-24]. Yet, the nature of virulence evolution in these organisms during chronic infection remains poorly understood. To assess how virulence evolves in an opportunistic pathogen during a chronic infection, we passaged *P. aeruginosa* PA14 in a murine chronic wound model for ten rounds of selection spanning 42 days, isolated a number of strains after the ten rounds for phenotypic and genotypic analysis, and compared the virulence of the whole evolved populations with that of the ancestor strain using a mouse sepsis experiment. We found that (i) there was a lower degree of morphological and phenotypic diversity in our evolved populations than has been previously described for *P. aeruginosa* in CF infections [27, 48, 49]; (ii) our populations acquired

mutations in major regulators and genes previously shown to be involved in pathogenesis; and (iii) virulence evolved differently in each of the three independent evolutionary lines.

To interpret these results and make meaningful predictions for human chronic infections, we must consider the following caveats: (i) time scale of our experiment, (ii) growth in the environment, and (iii) the number of isolates tested for phenotypic and genotypic analysis. The time scale of our experiment, 42 days, is significantly shorter than that of a human chronic wound infection. Additionally, we conducted generations of growth in LB medium in between rounds of evolution in mice, which may have introduced another variable of selection. We also acknowledge that while the sepsis experiment is a measure of population-level virulence, the virulence factor phenotypes, i.e. proteases, siderophores, pyocyanin, and motility, were conducted on a small sub-sample of isolates and may not be reflective of the population-level phenotype. Given all of these considerations, caution must be exercised when extrapolating experimental results from a laboratory mouse model to a vastly more complex human infection, as virulence factors can be host specific [66].

We first assessed morphotypic diversity within *P. aeruginosa* populations after ten rounds of selection. Previous studies on *P. aeruginosa* in the CF lung have shown a high degree of heterogeneity, with populations displaying up to 15 different morphotypes in a single patient's sputum sample [67, 68]. In our study, we only identified five distinct morphotypes which were smooth, non-mucoid, of similar size and with only small variations in pigment production. The low degree of morphotypic diversity we observed may be due in part to the time scale of our experiment, as six weeks is not comparable to the years of evolution in a CF lung. In addition, a chronic wound may lack the spatial structure seen in CF lungs [50], providing fewer niches for diversification. Further, our experiment focused on a monospecies infection, and CF lungs (and human chronic wounds) are comprised of polymicrobial infections, which may encourage further diversification through microbial interactions [69].

To understand how *P. aeruginosa* adapts to a chronic wound environment, we whole genome sequenced one isolate of each representative morphotype after ten rounds of selection. We identified mutations in *lasR*, *pvcA*, *rpoN*, *fleQ*, and *pilR*, all genes previously shown to be important for *P. aeruginosa* virulence [54-65]. The occurrence of the same *pvcA* and *lasR* mutations in all three lines presents evidence of parallel evolution, suggesting that these mutations may confer fitness advantages in wounds. LasR plays an important role in the quorum sensing (QS) hierarchy of *P. aeruginosa*, acting as a transcriptional activator for a plethora of genes implicated in virulence [57]. Although the in-frame deletion we observed in LasR, ΔS^{44}-D^{46}, is not at an active site, it is in extremely close proximity to a number of residues forming a ligand-binding pocket— G^{38}, L^{39}, L^{40}, Y^{47}, E^{48}, and A^{50} [70]. Furthermore, it is conceivable that a three-residue deletion could significantly impact protein folding and lead to a loss-of or decreased function. This is in agreement with the phenotypes we observed, as our *lasR* mutants showed decreased protease production and inhibited swarming ability, and it has been previously shown that *lasR* mutants show diminished swarming behavior [41]. PvcA is involved in the biosynthesis of paerucumarin, a molecule which has been suggested to enhance the expression of iron-regulated genes by chelating extracellular iron [64, 65]. The *pvcA* mutation we observed in all three lines ($A^{249}T$) of a non-polar to a polar side-chain amino acid, while not in an active site, could reasonably result in a detrimental impact to three-dimensional protein folding, and potential loss-of-function [71].

322
323 The mutations in *rpoN*, *fleQ*, and *pilR* all lead to amino acid changes within highly conserved
324 domains or residues directly involved in the activity of their respective protein products [72-74].
325 The gene product of *rpoN*, RNA polymerase factor σ^{54}, regulates a wide variety of functions in *P.*
326 *aeruginosa*, including the *rhl* QS system, flagellin, and pilin production, which play important
327 roles in motility, surface attachment, and biofilm formation [54, 55, 60-63]. Likewise, our *rpoN*
328 mutant, B42, showed inhibited motility. FleQ is a transcriptional regulator for both flagellin and
329 exopolysaccharide (EPS) biosynthesis in *P. aeruginosa* [58]. The *fleQ* mutants, C31 and C62,
330 displayed diminished swimming and swarming motilities. Lastly, PilR is a transcriptional
331 regulator for type IV pili expression, a structure involved in twitching motility and DNA uptake
332 [56]. While we did not phenotypically assess twitching motility, a mutation in a core functional
333 residue suggests most likely loss-of-function. The locations of these mutations, along with the
334 supporting phenotypic data, suggest that all of these mutations have led to loss-of-function. It has
335 previously been shown that in chronic CF infections, *P. aeruginosa* selects against the production
336 of virulence factors that are required for acute infection [27, 53]. Many CF isolates are *lasR*, *rpoN*,
337 and *fleQ* mutants [25-28]; likewise, another long-term evolution experiment of *P. aeruginosa*
338 found an accumulation of *lasR* and various *pil* mutants [75]. Our results, taken with previous
339 studies, suggest that *P. aeruginosa* may employ similar genetic adaptations in multiple infection
340 environments.
341
342 We found that evolution, with respect to levels of overall virulence, was not reproducible in our
343 three independent selection lines, in contrast to a previous study that showed *P. aeruginosa*
344 evolution was highly reproducible *in vitro* [76]. This highlights the likely importance of host-
345 specific variables such as the immune response on *P. aeruginosa* evolution and virulence. None
346 of the current classic virulence models (see introduction) in isolation adequately explains this
347 diverging pattern, suggesting there may be previously overlooked variables influencing virulence
348 evolution in opportunistic pathogens, or that components from multiple virulence models may
349 need to be considered in tandem. Heterogeneity within *P. aeruginosa* populations may partially
350 account for the differing virulence trajectories we observed, as within-host adaptation leading to
351 multiple infecting genotypes can result in higher or lower virulence, depending on the context.
352 Levin & Bull originally proposed the short-sighted evolution hypothesis to explain the role of
353 multiple infection and within-host selection on virulence [9]. According to their model, as a strain
354 mutates and diversifies within the host, competition for limited resources will favor fast-growing
355 genotypes, leading to an overall higher virulence. An alternative idea was proposed by Buckling
356 and West, where virulence is predicted to decrease in response to heterogeneity or low relatedness,
357 if virulence is dependent on the production of common goods and cooperation by members of the
358 population [77]. Such common goods are exploitable by non-cooperating 'cheats' that increase
359 their fitness in the population by benefitting from goods produced by cooperators, while
360 undermining the pathogenicity of the population as a whole [78-82]. We found some support for
361 both of these ideas, as we observed higher virulence in one evolutionary line but lower virulence
362 in another.
363
364 In *P. aeruginosa,* quorum sensing (QS) controls the production of a number of secreted common
365 goods, and strains isolated from chronic infections frequently display mutations in the QS regulator
366 *lasR.* We identified *lasR* mutations in four of our isolates, but it remains to be determined whether
367 they arose via social cheating or because they are better adapted to a wound environment.

However, their presence in our evolved populations is a plausible explanation for the reduction in virulence in one selection line, as the frequency of *lasR* mutants in populations has previously been linked to a reduction in social traits, virulence and antibiotic resistance [76, 79, 80, 82]. Another study on the evolution of *P. aeruginosa* in *Caenorhabditis elegans* also identified that virulence evolution was mediated by the production of secreted molecules. and that attenuation in pathogenicity could be attributed to cheats exploiting the goods produced by cooperators [75]. Taken together, this highlights the importance of social interactions during chronic infection, and that heterogenous populations likely result in complex interactions that can impact overall community function [76, 83]. Future work in this area could focus on exploring genetic diversity in infections using deep sequencing, which may provide insights as to how allelic polymorphism and genetic heterogeneity impacts community function and the outcome of virulence [76].

Overall, our study adds to the breadth of knowledge on *P. aeruginosa* adaptations *in vivo*, showing that while *P. aeruginosa* employs similar adaptive strategies, i.e. loss of virulence factors, in both chronic wounds and CF lungs, heterogeneity in chronic wounds may be less extensive. Our findings also emphasize that more work needs to be performed to increase our understanding of the dynamics and drivers of virulence evolution in opportunistic pathogens during chronic infection. This is an important consideration given the increasing interest in developing anti-virulence management strategies.

Data accessibility. All sequences have been uploaded to the NCBI SRA database (accession number PRJNA643594). Raw data, code, and Sanger sequencing results have been made available in the Dryad Digital Repository (https://doi.org/10.5061/dryad.000000021).

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.

Author contributions. SPD and KPR designed the study. JV and DF performed the experimental work and analyzed the data. All authors contributed to the writing of the manuscript.

Competing interests. The authors declare no competing interests.

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FIGURE & TABLE LEGENDS

Figure 1. *P. aeruginosa* population densities in wound bed and spleen tissues during serial passage experiment are positively correlated. (A) Wound bed CFUs for mice at time of death

414 for each evolutionary round were relatively stable, aside from the 8th mouse in line A. (B) Spleen
415 CFUs for mice at time of death for each evolutionary round were highly variable throughout the
416 experiment, with many values falling below our limit of detection (10^2 cells). Each CFU count
417 represents one technical replicate. Wound bed and spleen CFUs during the serial passage
418 experiment were positively correlated (Pearson's $r(28) = .44$, $p = 0.015$).
419
420 **Figure 2. Changes in morphology, protease production, and swimming and swarming**
421 **motilities.** (A) There were five distinct types of colony morphology on CRA at the final round of
422 selection across all three lines of evolution, with line A having two distinct morphology types, and
423 lines B and C each having three distinct colony morphology types (with some colony morphology
424 types being present in multiple lines). (B) Isolates A92, B16, C38, and C62 displayed protease
425 activity comparable to that of the ancestral PA14, while isolates A88, B31, B42, and C31 showed
426 decreased protease activity. (C) Isolates B42, C38, and C62 lost the ability to swim. (D) Isolates
427 A88, B31, B42, C31, C38, and C62 lost the ability to swarm.
428
429 **Figure 3. Changes in production of pyoverdine, pyochelin, and pyocyanin.** (A) Pyoverdine
430 production in the final evolved representative isolates and ancestral PA14. A88 was the only
431 evolved isolate with pyoverdine production significantly different than the ancestor strain
432 (Kruskal-Wallis, Dunn's post hoc test, Benjamini & Hochberg correction, $p = 0.018$). Error bars
433 indicate SEM. (B) Pyochelin production in the final evolved representative isolates and ancestral
434 PA14. A88 was the only evolved isolate with pyochelin production significantly different than the
435 ancestor strain (Kruskal-Wallis, Dunn's post hoc test, Benjamini & Hochberg correction, $p =$
436 0.0082). (C) Pyocyanin production in the final evolved representative isolates and ancestral PA14.
437 C38 and C62 both displayed pyocyanin production significantly different than the ancestor strain
438 (Kruskal-Wallis, Dunn's post hoc test, Benjamini & Hochberg correction, $p = 0.01975$ and $p =$
439 0.01915, respectively).
440
441 **Figure 4. Virulence can evolve in diverging directions in a chronic wound.** (A) Mice infected
442 by the final evolved population of line B in the sepsis experiment had the highest mortality rate
443 (100%), with no surviving mice at the end of 80 hours, while mice infected by line C had the
444 lowest mortality (40%), with three of five mice surviving. (B) Mice infected by the final evolved
445 population of line B in the sepsis experiment had significantly higher mean spleen CFUs at time
446 of death as compared to mice infected by line C, indicating more severe septicemia (Kruskal-
447 Wallis, Dunn's *post hoc* test, Holm-Bonferroni correction, $p = 0.023$). Error bars indicate SEM.
448
449 **Table 1.** A list of all mutations in the final evolved representative morphology type isolates as
450 mapped to the PA14 reference genome. Many genes coding for virulence factors or regulators of
451 virulence are mutated over the course of adaptation to chronic wounds.
452
453 **Supplemental Figure legends**
454
455 **Figure S1. Serial passage and sepsis experimental protocols.** (A) Serial passage experimental
456 protocol. We established three independent evolution lines by infecting three mice with ~10^3 cells
457 of the *P. aeruginosa* strain PA14 from a liquid lysogeny broth (LB) culture. Each infection
458 duration was 72 h, after which we euthanized the mice and harvested their wound bed and spleen
459 for colony forming unit (CFU) counts on *Pseudomonas* isolation agar (PIA). We used a 1:1000

460 serial dilution of the wound bed infection to start a new LB liquid culture and inoculate the next
461 mouse in each line of evolution, again with ~10^3 cells. We carried this selection experiment
462 through a total of ten passages in mice for each of the three parallel evolution lines (n=30 mice in
463 total). One replicate evolutionary line is shown in the diagram. (B) Sepsis experimental protocol.
464 We began the sepsis experiment by growing LB liquid cultures of the three final evolved
465 populations from the serial passage experiment and of the ancestral PA14. We used each of these
466 four liquid cultures to inoculate a distinct set of five mice with ~10^5 cells (n=20 mice in total). We
467 monitored these mice for 80 h for sepsis. If a mouse was moribund, it was euthanized, time of
468 death noted, and spleen harvested for CFU counts. At the end of 80 h, all remaining mice were
469 euthanized, and their spleens harvested for CFU counts.
470
471 **Table S1.** Details of the biofilm colony morphology types of evolved isolates on CRA after ten
472 rounds of selection spanning forty-two days across all three lines of evolution. One isolate of each
473 distinct colony morphology type from each population was selected as a representative for further
474 phenotypic and genomic analysis.
475

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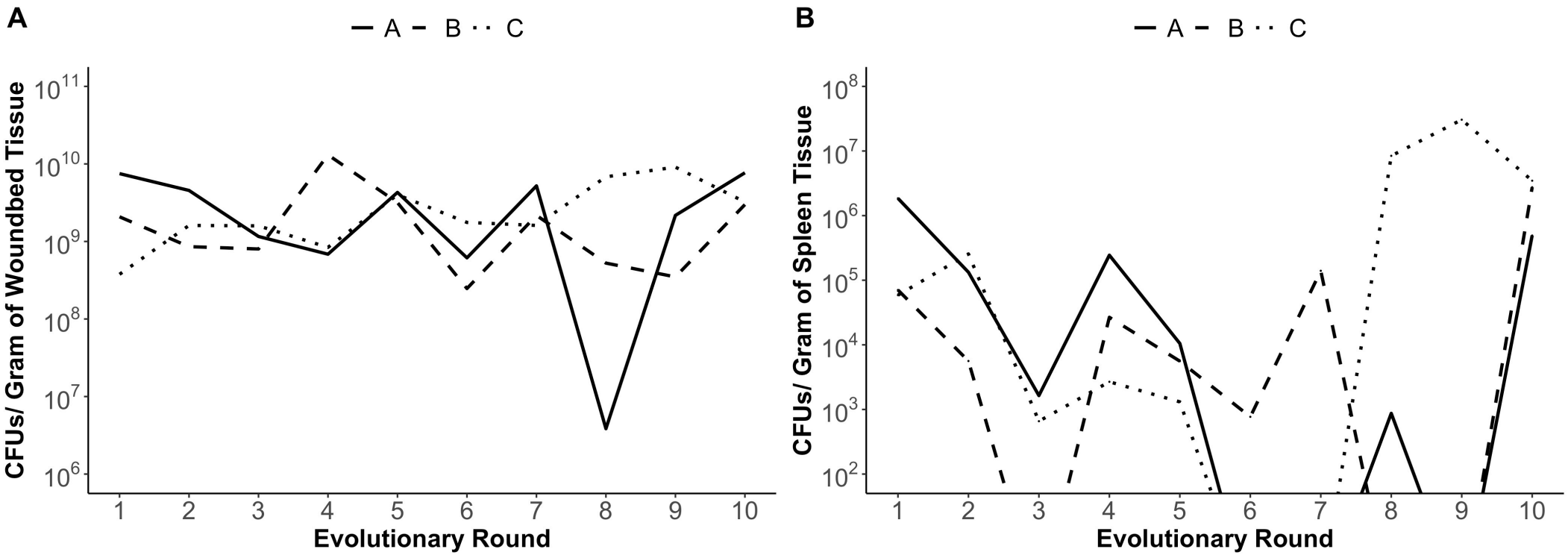
Figure 1


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

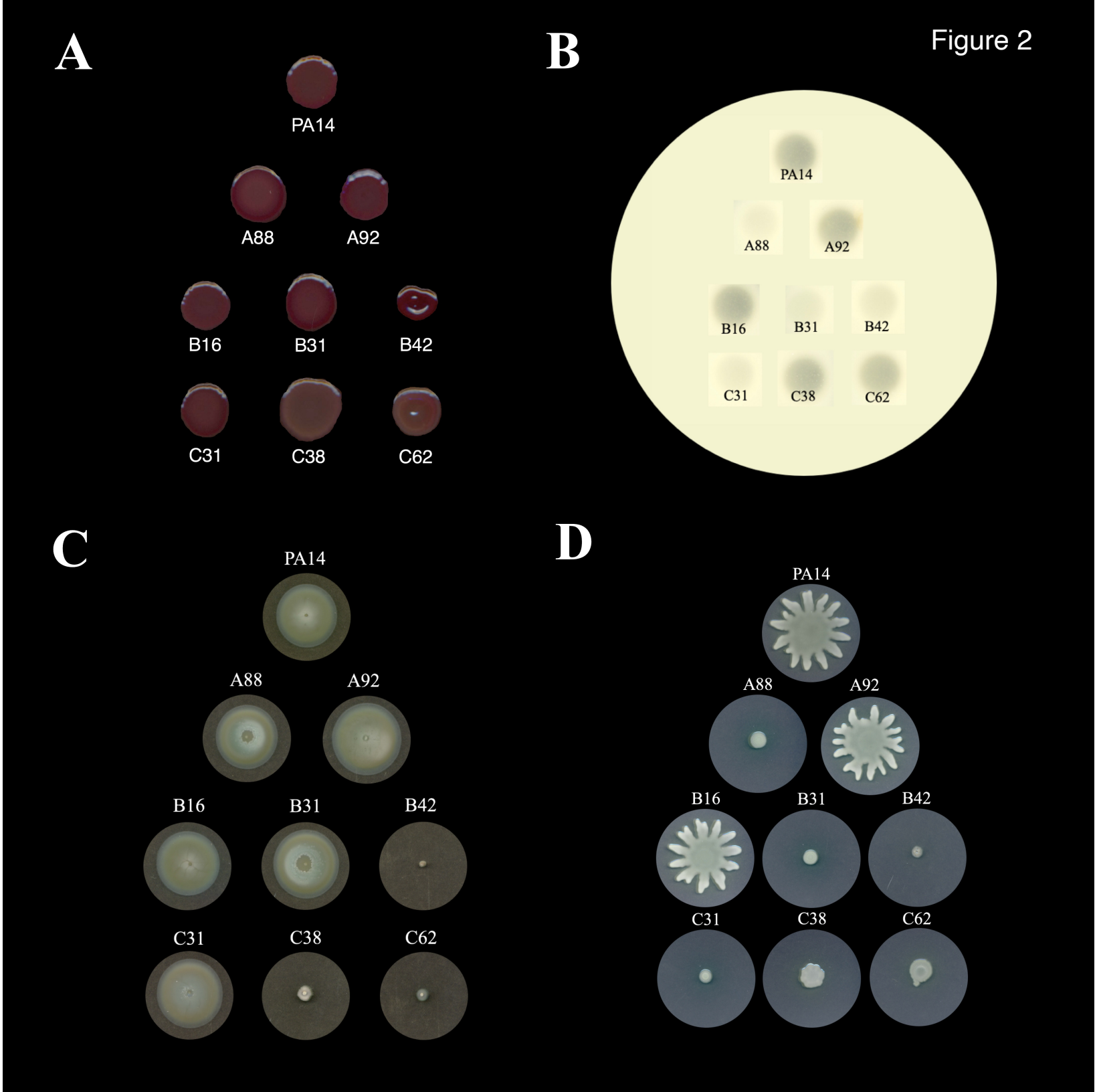


Figure 2
A
PA14
A88
A92
B16
B31
B42
C31
C38
C62
B
PA14
A88
A92
B16
B31
B42
C31
C38
C62
C
PA14
A88
A92
B16
B31
B42
C31
C38
C62
D
PA14
A88
A92
B16
B31
B42
C31
C38
C62

Fig 3

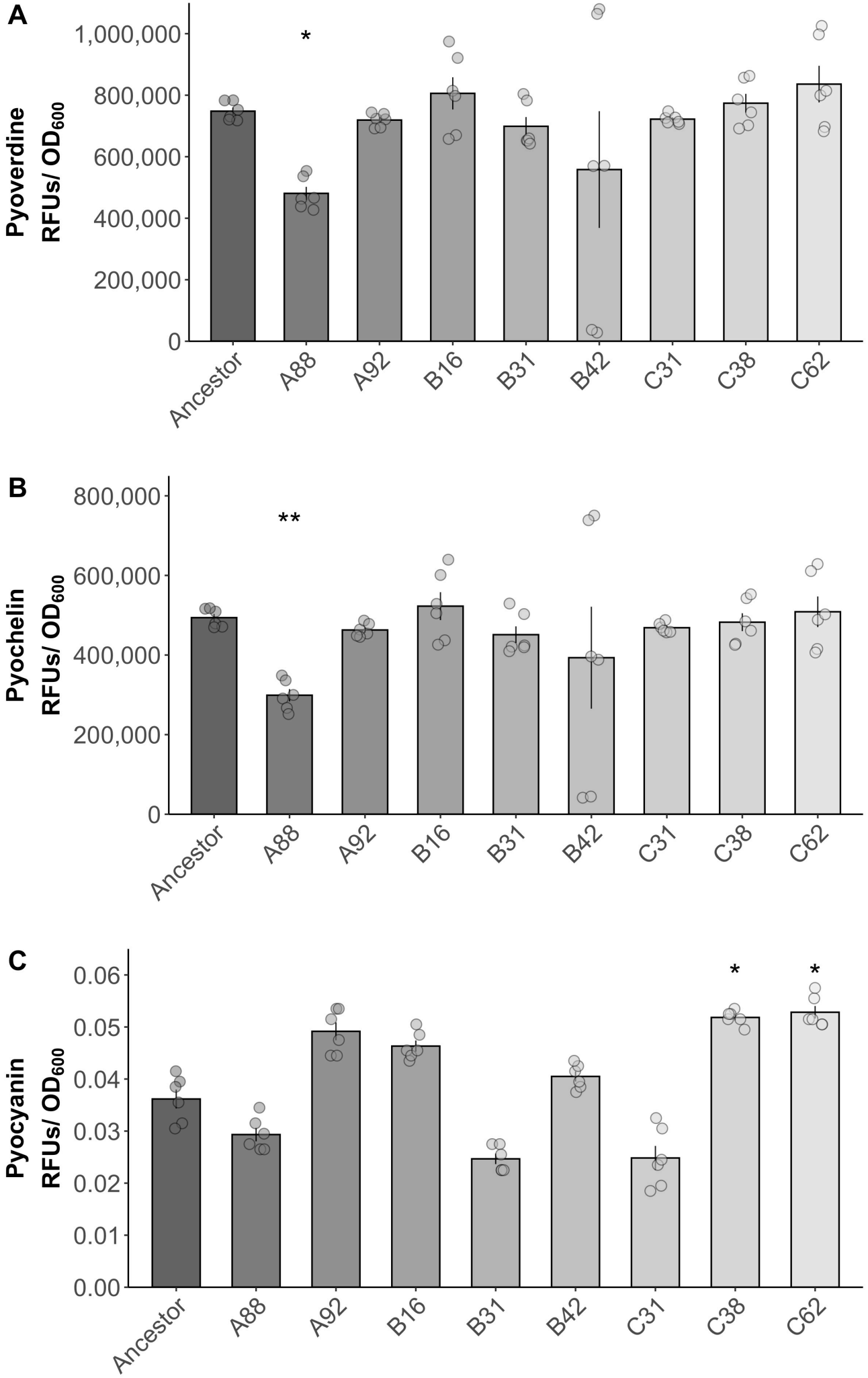

A
Pyoverdine
RFUs/ OD600
1,000,000
800,000
600,000
400,000
200,000
0
*
Ancestor
A88
A92
B16
B31
B42
C31
C38
C62
B
Pyochelin
RFUs/ OD600
800,000
600,000
400,000
200,000
0
**
Ancestor
A88
A92
B16
B31
B42
C31
C38
C62
C
Pyocyanin
RFUs/ OD600
0.06
0.05
0.04
0.03
0.02
0.01
0.00
*
*
Ancestor
A88
A92
B16
B31
B42
C31
C38
C62

Figure 4

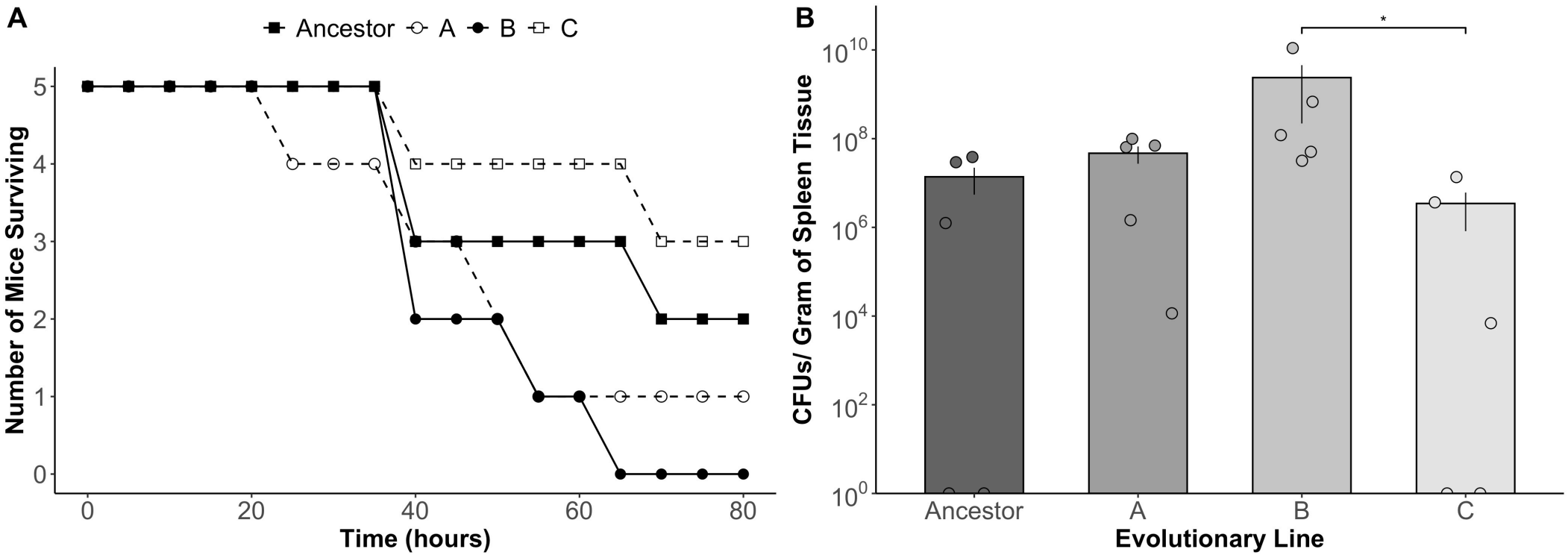

A
Ancestor
A
B
C
Number of Mice Surviving
0
1
2
3
4
5
0
20
40
60
80
Time (hours)
B
CFUs/ Gram of Spleen Tissue
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
10^{0}
*
Ancestor
A
B
C
Evolutionary Line

Table 1.

Gene locus	Gene annotation	Genetic mutation	Amino acid effect	Isolate(s)
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

Figure S1

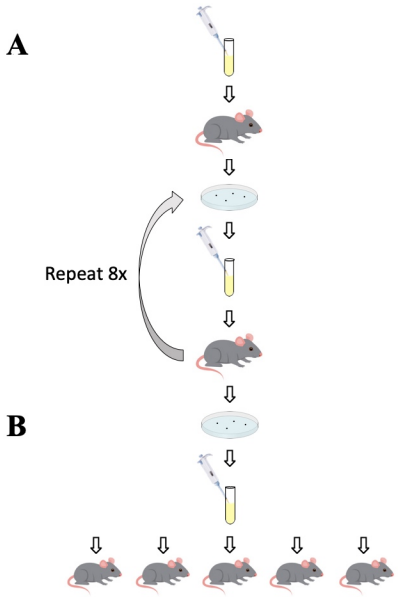


Table S1.

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