Predation maintains virulence in the opportunistic pathogen Serratia marcescens

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Abstract

Opportunistic pathogens are environmental microbes that are generally harmless and only occasionally cause disease. Unlike obligate pathogens, the growth and survival of opportunistic pathogens does not rely on host infection or transmission. Their versatile lifestyles make it challenging to decipher how and why virulence has evolved in opportunistic pathogens. The Coincidental Evolution Hypothesis (CEH) postulates that virulence results from exaptation or pleiotropy, i.e., traits evolved for adaptation to living in one environment that have a different function in another. Several studies have tested the CEH, but results have been inconclusive. Here we used experimental evolution to determine how the presence of a predator impacts the virulence and fitness of a ubiquitous environmental opportunistic bacterial pathogen that has acquired multi-drug resistance: *Serratia marcescens*. We evolved *S. marcescens* in the presence or absence of a generalist protist predator, *Tetrahymena thermophila*, and evaluated the genotypic and phenotypic changes that occurred after evolution. Overall, we found that evolution in the presence of a predator maintained virulence, whereas evolution in the absence of a predator resulted in attenuated virulence. Strains evolved in the presence or absence of a predator presented different phenotypic traits, such as increased biofilm formation and decreased predation resistance, respectively. Whole genome shotgun (WGS) sequencing of the evolved populations and isolates revealed multiple cases of parallel evolution in genes associated with virulence. Our results provide evidence that bacterial virulence and virulence related traits are maintained by selective pressures imposed by protist predators, supporting the CEH.

Introduction

Opportunistic pathogens are environmental bacteria that typically coexist peacefully with hosts and only sometimes cause disease, e.g., in hosts with immunodeficiency or microbiome imbalance [1]. Opportunistic pathogens are intriguing because they have diverse lifestyles and experience strong tradeoffs between adaptation to life in the environment versus life inside of a host. Opportunistic pathogens are also conceptually challenging because, unlike obligate pathogens, their survival does not depend on their ability to infect a host: they are fully capable of reproducing and living in the environment [1,2]. Thus, the factors driving virulence in opportunistic pathogens remain largely enigmatic from an evolutionary standpoint. One hypothesis that has been proposed for opportunistic pathogen virulence is the Coincidental Evolution Hypothesis (CEH) [3,4]. The CEH postulates that virulence evolves indirectly due to selection that occurs in non-host environments [3,4]. Specifically, bacterial defense mechanisms against environmental predators (e.g., amoebae and protists) such as adherence, biofilm formation, digestion resistance, intracellular toxin production, and outer-membrane structures have been shown to play a role in host infection [1,5,6]. Additionally, some studies have supported the CEH by showing that pathogens challenged with microbial predators exhibited increased host virulence [7–12], and genes involved in pathogenesis were upregulated during predation [11].

Experimental evolution represents a powerful approach for evaluating how predation impacts virulence. To date, the limited studies that have experimentally evolved bacteria in the presence of a predator have generally concluded that predation attenuates virulence [13–17]. However, it is difficult to make broad generalizations from these studies as they have used incomparable and often inadequate methods to assess phenotypes (e.g., testing entire evolved populations, mixtures of evolved isolates, or using different individual isolates for various assays). Moreover, to our knowledge, only one bacteria-predator experimental evolution study investigated the genotypes of the evolved bacteria and the authors claim to have identified over 100 mutations within their predator-evolved populations after only

three days of evolution and ~30 mutations (most at low frequency) in individual isolates after 42 days [17]. This high number of mutations [17] is highly unrealistic and is suggestive of contamination and/or sequencing error. Indeed, mutation accumulation experiments of bacteria have reported a range of 10^{-8} to 10^{-10} mutations/bp/generation, meaning that, on average, less than one mutation will arise in one generation in the absence of negative selection [18] and in the Long-Term Evolution Experiment only six mutations were observed in an evolved *Escherichia coli* clone (isolate) after 2,000 generations [19]. Therefore, the goal of our study was to experimentally evolve the opportunistic bacterial pathogen *Serratia marcescens* in the presence or absence of a ubiquitous environmental protist predator, *Tetrahymena thermophila,* and characterize both the genotypes and phenotypes of the evolved bacteria.

S. marcescens is an opportunistic pathogen that ubiquitously occurs in soil and water [20] and has been used for several previous bacteria-predator experimental evolution studies [13,14,16]. It is a host generalist that can colonize many different organisms, where it can be pathogenic and/or commensal [20]. In humans, *S. marcescens* is a common enteric bacterium that is generally harmless in the gastrointestinal tract [21]. However, it was recently found to be capable of damaging gut epithelial cells *in vitro* [22] and is responsible for causing dangerous nosocomial infections worldwide, notably in the respiratory and urinary tracts [23,24]. In the last few decades, the frequency of nosocomial infections caused by *S. marcescens* has increased, several hospital-wide outbreaks have been reported, and most strains have acquired multidrug resistance [23,24]. Additionally, *S. marcescens* has been recognized as an opportunistic pathogen of many plants and other animals [20]. Thus, understanding the evolution of virulence in *S. marcescens* is important for the health of many organisms.

Here, we evaluated whether experimental evolution of *S. marcescens* in the presence and absence of a predator could be useful for identifying genes involved in virulence and elucidating how

predation impacts opportunistic pathogen evolution. Following 60 days of experimental evolution in the presence or absence of *T. thermophila*, we sequenced random *S. marcescens* isolates from each population as well as the entire evolved populations and characterized the mutations that occurred. Using a single representative isolate from each evolved population we evaluated biofilm production, growth, and predation resistance to measure fitness and performed survival assays to test virulence in a natural animal host [25], the honey bee (*Apis mellifera*). Overall, our results revealed that virulence and grazing resistance is attenuated in the absence of a predator and demonstrated that predation plays a role in maintaining virulence in opportunistic pathogens, even over short evolutionary timescales. Additionally, we observed multiple cases of parallel evolution which, when combined with our phenotypic assays of isolates, allowed us to infer the outcome of some mutations. In conclusion, our study supports the CEH and provides evidence that experimental evolution can be used to identify genes involved in virulence and other phenotypes in *S. marcescens*.

Results

We evolved *S. marcescens* KZ19, isolated from honey bees (23), by serially passaging every day for 60 days (in Neff media [26]) in the presence or absence of a ubiquitous generalist protist predator *T. thermophila* (Figure 1). In our study we minimized predator/prey co-evolution by passaging evolved *S. marcescens* to new axenic *T. thermophila* cultures each day. In total, we created three media-evolved (ME) and six predator-evolved (PE) replicate lines (populations). The same concentration of *S. marcescens* was transferred for each passage, and we confirmed successful passaging via plating on LB agar every day. Viability and approximate population size of *T. thermophila* was evaluated before every passage via light microscopy. We also confirmed that grazing was occurring by co-culturing *T. thermophila* with transconjugant *S. marcescens* KZ19 containing an E2 crimson fluorescent protein with spectinomycin resistance [25,27] for 24 hours in Neff media with 180 ug/ml spectinomycin and then visualizing via fluorescent microscopy (Figure 1).

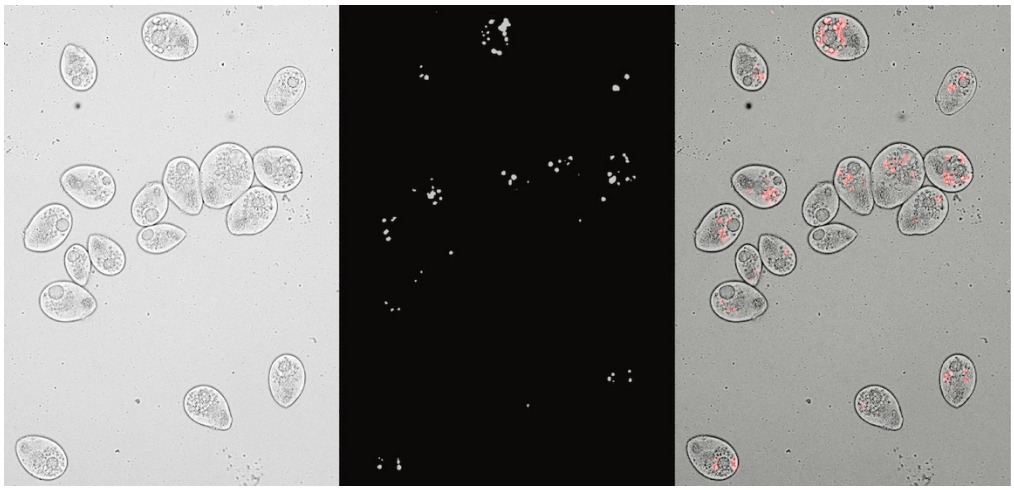

Figure 1. Confirmation of grazing activity. A) Phase contrast image of *T. thermophila* after 24h co-culture with fluorescently labeled *S. marcescens*, B) Cy5 filter image showing the E2 crimson labeled S. marcescens cells inside *T. thermophila*, and C) composite overlay of the phase contrast and fluorescent images. Images were taken at 20X on a Keyence BZ-X700 Series All-In-One Fluorescence Microscope and overlayed in ImageJ.

Following 60 days of evolution, the ancestral strain (n=1), PE populations (n=6), ME populations (n=3), and isolates from the three ME (n=10) and six PE (n=20) populations were sequenced using whole genome shotgun (WGS) sequencing. (Supplementary Table 1). We then characterized the virulence and fitness of one isolate from each evolved population (ME=3 and PE=6).

Mutations in the predator- and media-evolved populations

All evolved populations and isolates were compared to the ancestral genome to identify mutations. First, the sequencing reads of the ancestral strain were mapped to the reference KZ19 genome (23) to generate a consensus ancestral genome (average of 146X coverage; Supplemental Table 1). We then corrected for false positives by mapping all the reads of the ancestral strain back to the new consensus ancestral genome using BreSeq [28]. All mutations predicted via BreSeq [28] when the ancestral reads were mapped to the ancestral consensus genome were considered false positives if detected in the evolved populations (i.e., they most likely correspond to sequencing or assembly issues in the ancestral genome).

We first analyzed the mutation patterns across each population. The total number of mutations identified in each evolved population (ME n=3; PE n=6) ranged from 76 to 278 with an average of 85 mutations per population (Supplemental Table 1). The number of mutations present in a population was not correlated with the average genome coverage (P=0.16, R^2=0.26; Figure 2A) or with the average mutation frequency (P=0.18, R^2=0.25; Figure 2B). A lack of significant correlation between mutations and genome coverage reveals that our sequencing depth was sufficient to characterize the populations. Two PE populations that possessed over 230 mutations could be due to a lack of recent sweeps in these population or the presence of hyper mutators. We did find a significant negative correlation between average genome coverage and average mutations frequency (P=0.02, R^2=0.55; Figure 2C), indicating that sequencing depth impacted the number of low frequency variants detected.

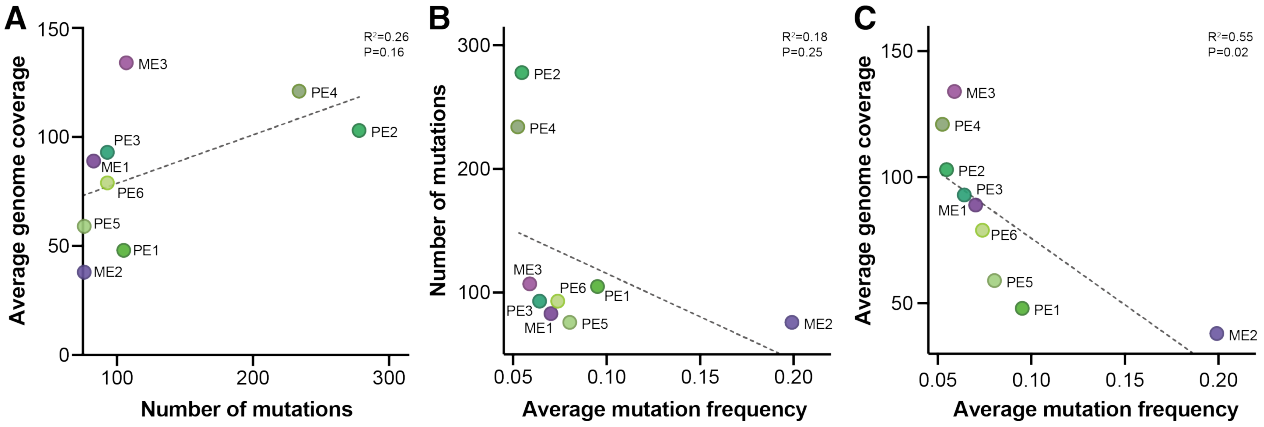

Figure 2. Comparison of **A)** the average genome coverage and the number of mutations detected, **B)** the number of mutations and the average mutation frequency, and **C)** the average mutations frequency and the average genome coverage for each evolved population.

In all evolved populations we identified nonsynonymous, synonymous, and intergenic mutations and a few mutations in non-coding regions (tRNAs) were observed in two ME and two PE populations (Figure 3A, Supplemental Dataset 1). Nonsynonymous mutations accounted for between 48-61% of the identified mutations in all populations. To determine the percentage of nonsynonymous, synonymous, intergenic, and non-coding mutations expected at random we introduced independent mutations into the ancestral *S. marcescens* KZ19 genome by performing 10,000 independent simulations using a

123 Jukes & Cantor model. We found that the percentage of nonsynonymous mutations we detected in our
124 populations was between 11-24% less than the random expectation which is 72.5% (Figure 3A),
125 indicating that both environments imposed negative (purifying) selective pressure on *S. marcescens*
126 (i.e., deleterious mutations are being purged).

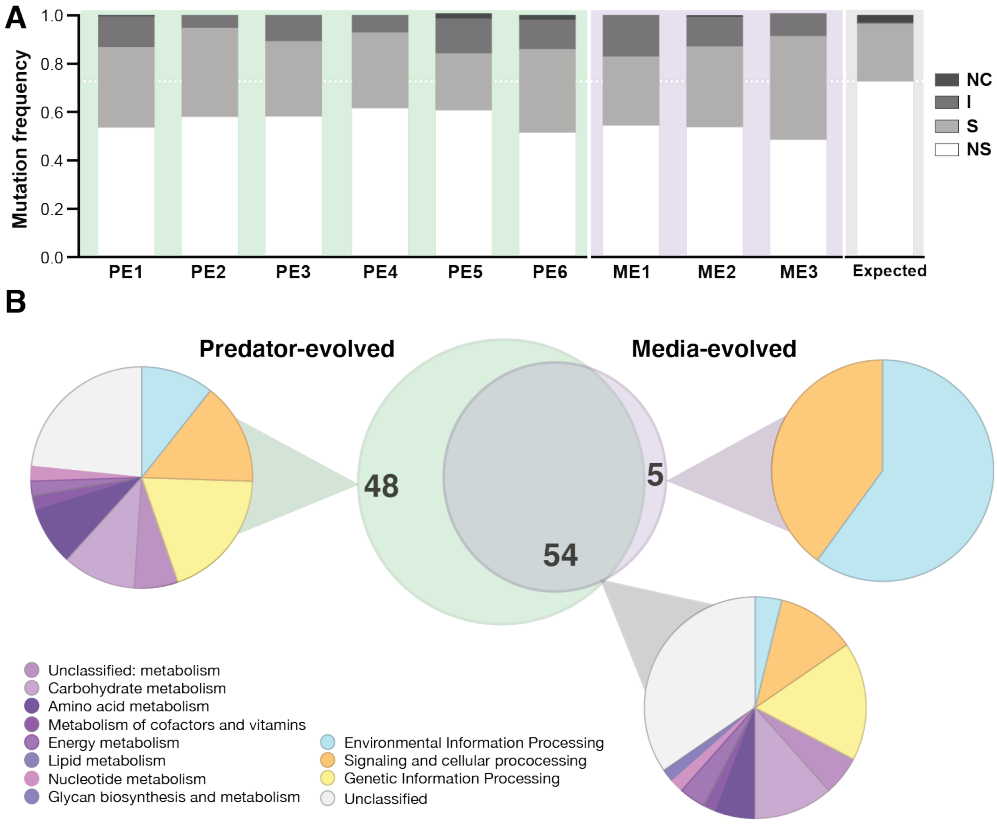

Figure 3. Characterization of the mutations present in the evolved populations. **A)** Percentage of nonsynonymous (NS), synonymous (S), intergenic (I), and non-coding (NC) mutations detected in each evolved population (PE and ME) and compared to the percentage of each mutation type expected at random (Expected). **B)** Number of parallel (*gene-* and *site-specific*) mutation events identified in only PE populations (n=48), only ME populations (n=5), or in both PE and ME (n=54) and the functional annotations of the genes in which parallel events occurred.

127 In addition to negative selection, we also found evidence that our populations were undergoing
128 positive selection based on the identification of numerous mutations either in the exact same position
129 (*site-specific* parallel evolution) or in the same gene (*gene-specific* parallel evolution) across
130 populations (Figure 3B, Supplemental Table 2). Parallel evolution events were considered if a mutation
131 was found in the same position or gene in independently evolved populations (ME, PE, or both). It is

132 important to note that none of the parallel evolution events occurred across all populations, confirming
133 that they were not present in the ancestral population. In total, 59 parallel evolution events occurred in
134 both ME and PE (PE+ME; n=54) or in only ME (ME; n=5) populations, suggesting that these mutations
135 are conferring adaptation to the Neff media. However, the parallel mutations only found in ME
136 populations are likely the result of lack of predation rather than adaptation to media. Most strikingly, 48
137 parallel evolution events were specific to the PE populations, indicating that these mutations/genes are
138 important for fitness in the presence of a predator. Of the total 107 parallel evolution events identified,
139 73% could be functionally classified (Figure 3B). Parallel mutations were identified in genes involved in
140 genetic information and processing (PE=19%, PE+ME=17%), signaling and cellular processes
141 (PE=15%, ME=40%, PE+ME=12%), environmental Information processing (PE=11%, ME=60%,
142 PE+ME=4%), and metabolism (PE=32%, PE+ME=33%).

143 *Mutations in the predator- and media-evolved isolates*

144 A total of 10 ME (3-4 per population) and 20 PE (1-5 per population) isolates were sequenced
145 from the evolved populations (Supplemental Table 3). All isolates were compared to the ancestral
146 genome to identify mutations using the same procedure applied to the entire populations (described
147 above and in the methods section).

148 At least one mutation was detected in each isolate, with a maximum of four and an average of
149 two mutations per isolate (Supplemental Table 3). We identified a total of 18 different mutations within
150 or upstream (in promoters) of 11 genes and 78% of the mutations (14/18) were present at a frequency
151 of >90% in their respective population (Supplemental Table 3). Most isolates from the same population
152 were clones or only differed by a single mutation, thus we chose one representative clone from each
153 population for further analysis (ME 1-3, and PE 1-6).

154 We plotted all the mutations identified in each ME and PE population and their observed
155 frequencies and found that our isolates possessed all the fixed (100% frequency) mutations present

156 within the population from which they were isolated (Figure 4A). Only three of the mutations present in

157 our isolates were detected at a frequency lower than 65% in their respective population (PE5: *yfiN,*

158 ME2: *barA*, and ME1: *rcsB*), but mutations at the exact same site or within the same gene occurred at

159 100% frequency in other populations (Figure 4A-B). In fact, we identified three instances of *site-specific*

160 parallel evolution and two cases of *gene-specific* parallel evolution across the isolates.

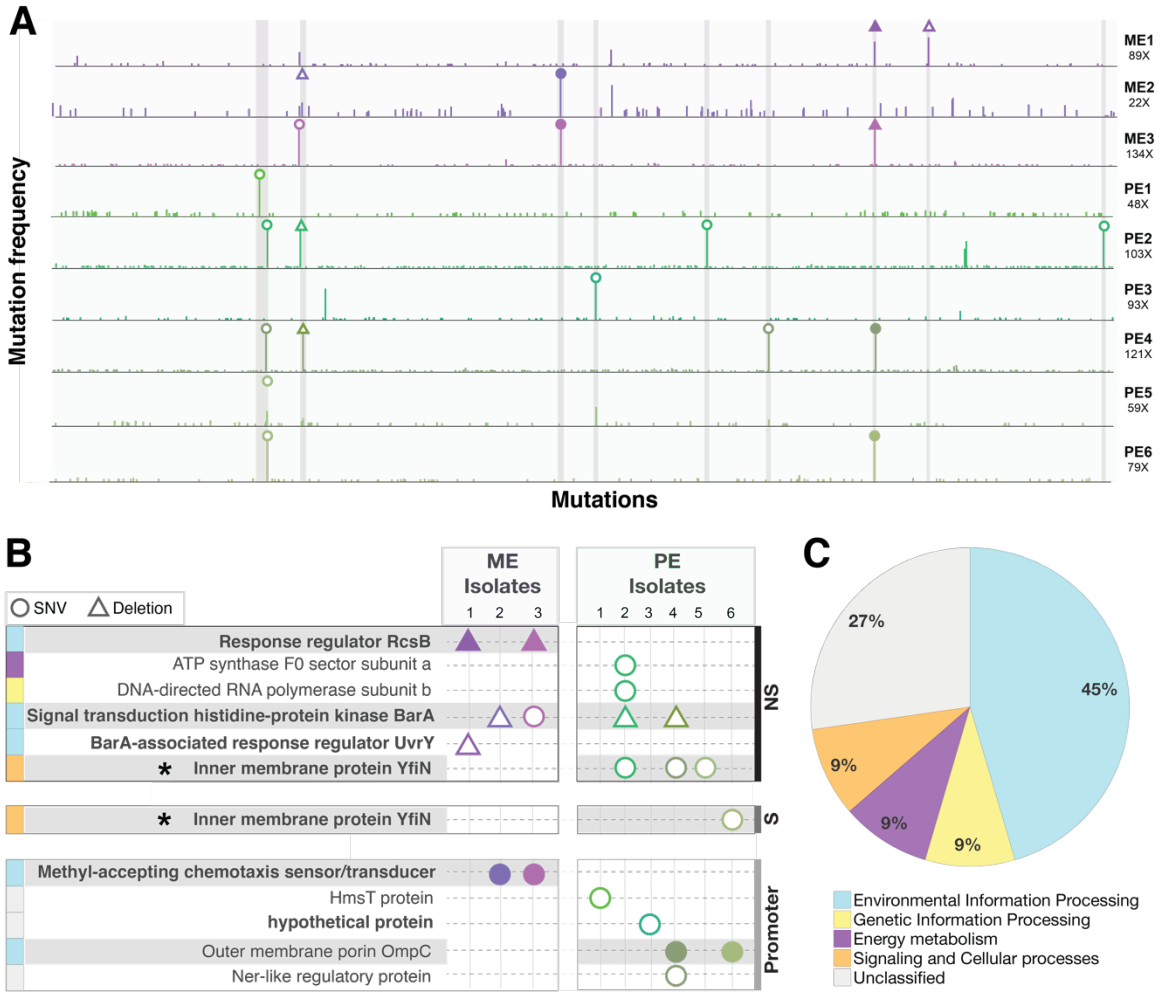

Figure 4. Mutations present in evolved isolates. **A)** Plot of all the detected mutations and their frequencies in each population highlighting the mutations present in each representative isolate (circles or triangles). **B)** Mutations present in each isolate grouped by nonsynonymous (NS), synonymous (S), promoter (intergenic mutation in a promotor region) and indicating the mutation type (single nucleotide variant (SNV) or deletion), the gene (or gene upstream) affected, and the functional category of the gene (left color boxes). Gray shading of a row highlights parallel evolution events (*site-* or *gene-specific*) and solid shapes denote cases of *site-specific* parallel evolution. **C)** Pie graph showing the functional classification of the genes (or genes upstream) impacted by mutation events in the individual isolates and the percentage belonging to each category.

161 The most striking case of *site-specific* parallel evolution was a 66 bp in-frame deletion in the
162 response regulator *rcsB* [29] present in the ME1 and ME3 isolates (Figure 4B). The two other *site-*
163 *specific* parallel evolution events occurred in promoters directly upstream of 1) a methyl-accepting
164 chemotaxis sensor/transducer protein in the ME2 and ME3 isolates and 2) the outer membrane porin
165 OmpC protein in the PE4 and PE6 isolates (Figure 4B). One of the *gene-specific* parallel evolution
166 events occurred in a gene annotated as *barA* (a signal transduction histidine-protein kinase) in the
167 ME2, ME3, PE2, and PE4 isolates (Figure 4B). The second case of *gene-specific* parallel evolution
168 arose in a gene annotated as the inner membrane protein *yfiN* in all PE isolates except PE1 and PE3
169 (Figure 4B). Cases of parallel evolution found across PE and ME lines suggest adaptation to the media,
170 whereas parallel evolution events unique to either PE or ME lines suggest adaptation to the presence
171 or to the absence of predation, respectively.

172 We were able to functionally characterize 73% (8/11) of the genes (or genes adjacent to the
173 promoter region) in which mutations occurred (Figure 4B-C). The eight characterized genes belonged
174 to four functional classes: environmental information processing (45%), genetic information processing
175 (9%), energy metabolism (9%), and signaling and cellular processes (9%) (Figure 4C). Several genes
176 in which we observed mutations have been associated with virulence factors in *S. marcescens* or
177 related enterobacteria, such as *yfiN* [30,31], *rcsB* [32,33], and *barA* and its response regulator *uvrY*
178 [34–36]. Additionally, all intergenic mutations identified in the isolates are located within promoters
179 upstream of five different genes (Figure 4B), some of which have been associated with biofilm
180 formation (HmsT protein [37]) and chemotaxis (Methyl-accepting chemotaxis sensor/transducer [38]).

181 <u>*Virulence of predator- and media-evolved strains*</u>

182 *S. marcescens* KZ19 is pathogenic in honey bees under certain circumstances, e.g., following
183 antibiotic treatment, when exposed to high doses, or when injected into the hemolymph [25]. Thus, we
184 determined how predation impacts the virulence of *S. marcescens* by performing survival assays in

honey bees via high dose exposure. We tested the virulence of one isolate from each predator-evolved population (n=6) and one isolate from each media-evolved population (n=3) by comparing them to the virulence of the ancestral strain (Figure 5A-B). Three assays (biological replicates) were done with five technical replicates each, totaling 300 bees per isolate.

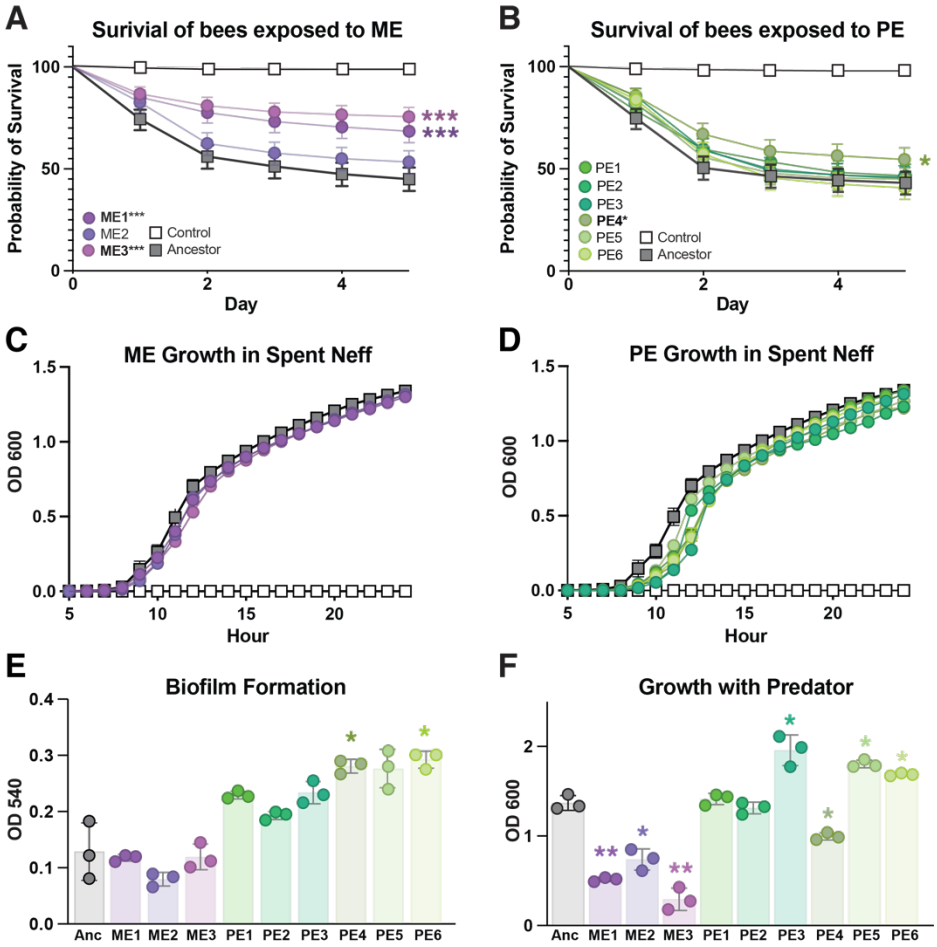

Figure 5. Virulence and fitness of evolved isolates. **A-B)** Probability of survival of honey bees exposed to the ancestral strain (black and gray) and the **A)** ME isolates and **B)** PE isolates. Control bees were given sterile sugar water only (white squares). Mortality was recorded each day for 5 days. Graphs are based on three replicate assays per isolate (n=300 bees per isolate). * = P<0.05, *** = P<0.0005; Mantel-Cox Log-rank test with Bonferroni correction. **C-D)** Mean growth rate of each variant based on 600nm OD readings every hour for 24h of **C)** ME and **D)** PE isolates in spent Neff media compared to the ancestral strain (black). **E)** Mean biofilm formation of ME and PE isolates and the ancestral strain based on 560nm OD readings after 36h of growth in Neff media. Error bars show the standard deviation between triplicates. * = P<0.05; *t*-test with Bonferroni correction. **F)** Mean predation resistance of PE and ME isolates and the ancestral strain based on 600nm OD readings of bacterial filtrate after 48h in co-culture with *T. thermophila*. Error bars show the standard deviation between triplicates. * = P<0.05, ** = P<0.005; *t*-test with Bonferroni correction.

189 All the ME isolates displayed attenuated virulence when compared to the ancestral strain, with
190 2/3 being significantly less virulent (Figure 5A; $P<0.001$, Mantel-Cox Log-rank test with Bonferroni
191 correction). Conversely, none of the PE isolates significantly differed from the ancestor in terms of
192 virulence, except for PE4 which was statistically less virulent than the ancestral strain (Figure 5B;
193 $P=0.007$, Mantel-Cox Log-rank test with Bonferroni correction). Overall, results show that in the
194 absence of predation, virulence is attenuated, and predation maintains virulence in *S. marcescens*.

195 *Fitness of predator- and media-evolved strains*

196 To test the fitness of the PE and ME strains, we performed growth, biofilm formation, and predation
197 resistance (population size after growth in the presence of *T. thermophila*) assays. For growth rate, we
198 conducted *in vitro* assays using three different media types A) spent Neff media (filtered media that had
199 supported *T. thermophila* growth for 24h; Figure 5C-D), B) fresh Neff media and C) LB broth. In all
200 media types, virtually all the ME and PE isolates displayed slightly slower growth than the ancestor over
201 24h, but in general, the growth of the PE variants was more attenuated than the ME variants (Figure
202 5C-D, Supplemental Figure S1). This pattern suggests a tradeoff between growth rate and resistance to
203 predation.

204 Because biofilm formation is a strategy for resistance to predation and is also involved in
205 virulence, we measured biofilm production in our ME and PE variants compared to the ancestor (Figure
206 5E). Overall, the ME isolates did not differ ($P=0.22$, Kruskal-Wallis test) from the ancestor in terms of
207 biofilm production, whereas the PE isolates produced significantly more biofilms than the ancestor
208 ($P=0.01$, Kruskal-Wallis test). In fact, biofilm production increased between 1.5- to 2.3-fold across PE
209 isolates relative to the ancestor (Figure 5E), indicating that they are producing more biofilms due to
210 predation. However, only two of the PE isolates were found to significantly produce more biofilms than
211 the ancestor when tested individually and corrected for multiple comparisons (PE4 $P=0.03$, PE6
212 $P=0.02$, *t*-test with Bonferroni correction).

213 Lastly, we evaluated predation resistance (i.e., growth with predator) of the ME and PE isolates.
214 Each isolate was grown with *T. thermophila* for 48h and then the population density of the bacteria was
215 measured (Figure 4F). In general, predation resistance of the PE isolates significantly differed from that
216 of the ancestor (P=0.01, Kruskal-Wallis). However, predation resistance across PE isolates differed
217 with some being similar to the ancestor (PE1 P=2.25 and PE2 P=1.8), whereas others were more
218 resistant (PE3 P= 0.026, PE5 P= 0.0063, and PE6 P= 0.0135) or less resistant (PE4 P= 0.0099) to
219 predation relative to the ancestor (Figure 4F; *t*-test with Bonferroni correction). Overall, the ME isolates
220 were significantly less resistant to predation than the ancestor (Figure 4F; P<0.0001; Kruskal-Wallis
221 test). All ME isolates were found to be significantly more susceptible to predation than the ancestor
222 when tested individually and corrected for multiple comparisons (Figure 4F; ME1 P= 0.001, ME2 P=
223 0.007, ME3 P= 0.001, *t*-test with Bonferroni correction). Results suggest that lack of predation
224 decreases grazing resistance while adaptation to predation increases it. Findings also indicate that the
225 PE lines followed different evolutionary paths over the 60 days of evolution.

226 **Discussion**

227 Evolution of *S. marcescens* in the absence of a predator resulted in attenuated virulence when
228 compared to the ancestral strain. Conversely, *S. marcescens* that were evolved in the presence of a
229 predator were not more virulent than the ancestral strain. Thus, our results indicate that virulence is
230 quickly attenuated in the absence of predation but is maintained in the presence of predation over short
231 evolutionary time scales. Maintenance rather than increased virulence following evolution with a
232 predator could be due to the fact that the ancestral strain (KZ19) is a natural opportunistic pathogen of
233 honey bees [25] and may have limited potential to become more virulent, especially during only 60 days
234 of evolution and because of the protection provided by the native gut microbes [25,39].

235 We found that the growth rate of PE and ME isolates did not substantially differ from the
236 ancestor. Nevertheless, growth rate of the PE isolates was systematically lower than the ME isolates,

237 likely revealing some fitness tradeoffs involved in adaptation to predation. Biofilm formation was
238 increased in the PE isolates when compared to the ancestor and the ME isolates. Biofilm production is
239 a natural defense mechanism against predation and can also play a role in pathogenicity [40]. Only half
240 of the PE isolates displayed a larger population size than the ancestor after 48h of co-culture with *T.*
241 *thermophila* and one PE isolate, PE4, was significantly less resistant to predation than the ancestral
242 strain, revealing that evolution in the presence of a predator does not always result in increased
243 resistance to predation. However, the predation-susceptible PE4 isolate also showed attenuated
244 virulence in honey bees, suggesting a link between predation resistance and virulence. We found that
245 all ME isolates were significantly less fit in the presence of a predator than the ancestor, indicating that
246 evolution in the absence of predation reduces fitness in the presence of a predator. Moreover, the ME
247 isolates that displayed attenuated virulence were also the most susceptible to predation (i.e., had the
248 smallest population size after co-culture with *T. thermophila*). Together these results imply that
249 virulence and predation resistance are governed by the same mechanisms. It is important to note that
250 we observed phenotypic differences after only 60 days of evolution and with only one to four identified
251 mutations per isolate.

252 Several different mutations, including some in genes associated with virulence, arose in the ME
253 and PE isolates. Most mutations observed were non-synonymous or located in promoters, one
254 synonymous mutation was also identified. Multiple cases of parallel evolution (site-specific and gene-
255 specific) were observed, indicating that some genes are under strong selection in the presence and/or
256 absence of a predator. With the exception of one synonymous mutation in *yfiN* in the PE6 isolate, most
257 instances of parallel evolution involved non-synonymous or intergenic mutations within promoters, both
258 of which likely impact gene expression and/or function. Though generally thought to be “silent”, recent
259 studies have revealed that synonymous mutations can have an impact on gene expression [41,42].
260 Therefore, the synonymous mutation that occurred in *yfiN* could have an impact on fitness, especially

261 because it was fixed in the PE6 population. In fact, virtually all of the parallel mutations observed in the
262 isolates were fixed within their respective populations.

263 A gene-specific parallel evolution event occurred in a gene annotated as *barA* in both ME and
264 PE isolates. In *E. coli*, *barA* encodes a protein part of a two-component system which plays an
265 important role in the ability to switch between different carbon sources [34]. Additionally, orthologs of
266 *barA* have been associated with virulence in several Gram-negative pathogens [34–36]. The mutation
267 in *barA* present in PE4 most likely resulted in a loss of function since it involved a 1bp frameshift
268 deletion. In other isolates, it is likely that the mutations altered the function or expression of *barA* since
269 they consisted of a nonsynonymous SNVs or in-frame deletions. Based on the evidence that *barA* is
270 involved in a carbon storage regulatory system in *E. coli* [34] and that mutations in this gene arose in
271 both PE and ME isolates, we predict that they are the result of adaptation to the Neff media, which
272 consists of proteose peptone, yeast extract, and glucose.

273 We also identified cases of parallel evolution that were specific to either ME or PE variants,
274 signifying that they are likely due to adaptation to the presence or to the absence of a predator rather
275 than the media. For example, in the isolates from ME Lines 1 and 3, the same 66bp in-frame deletion
276 (site-specific parallel evolution) occurred in a gene annotated as *rcsB*. RcsB is the response regulator
277 of the Rcs phosphorelay two-component signal transduction system in enterobacteria [32]. RcsB has
278 been associated with the synthesis of biofilms, capsules, and flagella and antibiotic resistance [32].
279 Isolates that contained the deletion in *rcsB* displayed significantly attenuated virulence and had much
280 lower population sizes after co-culture with *T. thermophila* than the ancestral strain, indicating that *rcsB*
281 plays a role in predation resistance and pathogenicity in *S. marcescens*. One other case of site-specific
282 parallel evolution found in ME2 and ME3 isolates was an intergenic mutation in the promoter upstream
283 of a methyl-accepting chemotaxis sensor/transducer protein. Methyl-accepting chemotaxis proteins are
284 used to sense and respond to environmental stimuli and are involved in the synthesis of biofilms,

285 flagella, and toxins in many pathogens [38]. The fact that this mutation was in a promoter and found in
286 isolates from different evolved lines suggests that it confers a fitness benefit in the absence of
287 predators, potentially by saving resources by reducing the expression or protein complexes.

288 In most of the PE isolates, a non-synonymous (PE2, PE4, PE5) and synonymous (PE6) SNV
289 was found in a gene encoding the inner membrane protein YfiN (gene-specific parallel evolution). YfiN
290 has been implicated in biofilm formation, inhibiting motility, and cell division arrest under stressful
291 conditions [30,31]. The fact that mutations arose in *yfiN* independently across different PE populations
292 and were often fixed (100% frequency) implies that this gene is under strong selective pressure in the
293 presence of predators. Mutations in *yfiN* were present in four of the six PE isolates and we speculate
294 that they are responsible for the observed increase in biofilm production. The PE variants that did not
295 acquire a mutation in *yfiN* either contained a mutation in a promoter directly upstream of 1) a
296 hypothetical protein (unknown function) or 2) the inner membrane protein HmsT. HmsT is associated
297 with biofilm formation in *Yersinia pestis* [37], one of the closest known relatives of *S. marcescens*.
298 Although only a speculation, we predict that the intergenic mutations in the promoter regions upstream
299 of HmsT and the hypothetical protein are involved in increasing biofilm formation in the PE1 and PE3,
300 respectively, as these were the sole mutations present in these isolates. In addition, PE3 displayed
301 significantly increased predation resistance, suggesting that the mutation in the promoter region
302 upstream of the hypothetical protein plays a role in defense against predators.

303 In conclusion, our results suggest that predation helps maintain virulence in opportunistic
304 pathogens, thereby supporting the CEH. We identified several instances of parallel evolution across
305 evolved populations, which, when combined with our phenotypic assays of isolates, allowed us to infer
306 the outcome of some mutations. For example, we found evidence that *barA* Is involved in carbon
307 utilization in *S. marcescens*. We also predict that the mutations we observed in *yfiN* and two promoters
308 (upstream of HmsT and of a hypothetical protein) are responsible for increased biofilm production.

309 Moreover, our results imply that RscB plays a major role in predation resistance and virulence in *S.*
310 *marcescens*. Fundamentally, our study demonstrates the efficacy of using experimental evolution to
311 identify virulence mechanisms, even over short evolutionary timescales. Surprisingly, we found that
312 evolution in the absence of a predator yielded the most informative results for identifying factors
313 associated with virulence. Performing long-term evolutionary experiments under different scenarios with
314 additional replicate populations will provide a more accurate picture of the forces that drive the
315 evolution of opportunistic pathogens and the mechanisms responsible for their virulence. Identifying
316 virulence mechanisms and understanding how virulence evolves can help guide the development of
317 more effective treatment strategies for combating bacterial infections.

318 **Materials and Methods**
319
320 *Microscopy*
321 To confirm that *T. thermophila* grazes on *S. marcescens* KZ19 we utilized transconjugant KZ19
322 containing an E2 crimson fluorescent protein with spectinomycin resistance. The transconjugant KZ19
323 strain was inoculated into 3mL of LB broth containing 180ug/mL spectinomycin and incubated at 30°C
324 for 24h. After 24h, 1OD of the transconjugant KZ19 was diluted to 10^{-5} in Neff media and 1mL was
325 pipetted into 3mL of Neff media containing *T. thermophila* and 180ug/mL spectinomycin. The culture
326 was incubated for 24h at 30°C. One mL of the 24h co-culture was then centrifuged at 2000g for 10m to
327 collect the *T. thermophila* cells, the supernatant containing bacteria was discarded, and the *T.*
328 *thermophila* were resuspended in 1mL of axenic Neff media. The *T. thermophila* cells were then
329 pipetted onto a microscope slide that was washed with 60% EtOH (which immobilizes the cells without
330 immediately lysing them), covered with a glass cover slip, and imaged on a Keyence BZ-X700 Series
331 All-In-One Fluorescence Microscope in brightfield and with a Cy5 filter.

332 *Experimental evolution*

333 *Serratia marcescens* strain KZ19 was evolved either in media alone (media-evolved) or with the
334 predator *Tetrahymena thermophila* strain SB210 (predator-evolved). *T. thermophila* strain SB210 was
335 purchased from the Tetrayhema Stock Center located at Cornell University. The media-evolved (ME)
336 experiment included three replicate lines and the predator-evolved (PE) experiment included six
337 replicate lines. First, *S. marcescens* and *T. thermophila* were cultured for 24h at 30°C in Neff media. To
338 start each evolved line, 1OD of the overnight KZ19 culture was diluted to 10^{-5} in fresh Neff media and
339 1mL was added to either 3mL of Neff media [26] alone (ME lines) or 3mL Neff media containing equal
340 concentrations of 24h *T. thermophila* culture (PE lines). The starting ratio of S. marcescens:*T.*
341 *thermophila* was based on preliminary trials starting with various concentrations of *S. marcescens* and
342 *T. thermophila* co-inoculated in Neff media to determine the ratio that would allow both organisms to
343 remain in co-culture for 24h.

344 The experimental cultures were incubated at 30°C without shaking. Every 24h for 60 days the
345 cultures were vortexed and 10μL of each culture was sampled from the flasks and added to 3mL of
346 either 1) fresh Neff media or 2) new axenic cultures of *T. thermophila* in Neff media. Every day, prior to
347 passaging, the cultures were checked for *T. thermophila* viability and density via microscopy and the
348 successful passaging and growth of *S. marcescens* presence was evaluated by plating on Luria-Bertani
349 (LB) agar. Every day over the course of the 60-day evolution experiment *T. thermophila* and *S.*
350 *marcescens* were always detected and we never observed a noticeable difference in population size
351 based on plating (*S. marcescens*) and light microscopy (*T. thermophila*).

352 On day 60, six LB agar plates were streak inoculated from the culture flasks and incubated for
353 24h at 30°C. Twenty bacterial colonies were randomly selected from the PE lines (1-5 per population)
354 and ten bacterial colonies were randomly selected from the ME lines (3-4 per population). Pure cultures
355 of each isolate (30 total) were created by inoculating in 3mL LB broth with 24h incubation at 30°C.
356 Samples (200μL from each pure culture) were then frozen at -80°C in 20% glycerol (*T. thermophila* do

357 not survive freezing). These 30 isolates were sequenced, analyzed, and one isolate from each evolved
358 population (ME n=3, and PE n=6) was used for phenotypic analysis (i.e., virulence, growth, biofilm
359 production, and predation resistance).

360 *Virulence assays*

361 Pure 1OD cultures of the evolved isolates and the ancestor (10 total) were pelleted via
362 centrifugation and resuspended in a 1:1 in sterile sugar syrup solution (SSS). Conventional adult honey
363 bee workers (*Apis mellifera*) were sampled from a single hive located on the Gateway North Research
364 Campus in Browns Summit, North Carolina. Bees were immobilized at 4C, randomly distributed into
365 groups, and exposed via the immersion method (23) to ~10μL of one of three treatments: 1) sterile SSS
366 only, 2) the ancestor in sterile SSS, or 3) each evolved isolate in sterile SSS. Bees were kept in cup
367 cages (20 bees/cup; 100 bees/treatment) under hive conditions: 35°C and 95% humidity. Mortality
368 between the treatments was noted every day for 5 days. Assays were performed in triplicate with 300
369 bees tested in total per isolate. Survival curves (Kaplan-Meier) were created in GraphPad Prism version
370 9.1.0.

371 *Fitness assays*

372 Growth assays were performed in triplicate in both LB broth, fresh Neff media, and spent Neff media
373 with *T. thermophila* filtered out (i.e., *T. thermophila* were present in the media for 24h before filtering).
374 Pure 1OD cultures of the evolved isolates and the ancestor were diluted to 10^{-5} and 5μL of the culture
375 was pipetted into 200μL of media in a 96-well microplate. Absorbance detection was performed at an
376 OD of 600nm at 30°C with shaking every hour for 24h on a BioTek Synergy™ 2 plate reader.

377 Biofilm assays were performed in triplicate for each isolate. Pure 1OD cultures of the evolved
378 isolates and the ancestor were diluted to 10^{-5} and 5μL of the culture was pipetted into 200μL of media
379 in a 96-well microplate. The covered plate was incubated for 36h at 30°C without shaking. After 36h,

380 the supernatant was poured off, the wells were washed twice with 200μL of sterile ddH_2O, and the cells
381 were stained with 150μL of 0.04% crystal violet for 10 minutes. After 10 minutes, the excess crystal
382 violet was removed, the wells were washed again and stained once more for 10 minutes. The crystal
383 violet was poured off and cells were solubilized with 150μL of 95% ethanol. OD readings were taken at
384 absorbance reading 560nm on the BioTek Synergy™ 2 plate reader.

385 Predation resistance assays were performed in triplicate for each isolate. Pure 1OD cultures of
386 the evolved isolates and the ancestor were diluted to 10^{-5} and 1mL was added to a culture flask
387 containing *T. thermophila* in 3mL Neff media. *T. thermophila* alone in 3mL Neff media served as the
388 negative control. Experimental culture flasks were incubated at 30°C for 48h. After 48h, the cultures
389 were mixed thoroughly and 1mL was sampled and centrifuged at 5,000 rpm for 10 minutes. The
390 supernatant was discarded, pellets were resuspended in 1mL 1X PBS, and the solution was filtered
391 through 5.0 μm MilliporeSigma™ Millex™-SV Sterile PVDF syringe filters to remove the *T. thermophila*
392 cells. OD readings were taken of filtrates at 600nm after blanking with the negative control filtrate.

393 *DNA Extraction and Sequencing*

394 Cultures of the evolved populations (ME n=3, PE n=6), evolved isolates (ME n=10, PE n=20) and the
395 ancestor (n=1) were diluted to 1 OD. DNA extractions were performed using the Zymo Quick-DNA
396 Fungal/Bacterial Miniprep Kit (D6005). The DNA was then prepared for Whole Genome Shotgun
397 (WGS) sequencing using the Illumina Nextera DNA Flex Library Prep Kit (20018704). Genomes were
398 sequenced in-house on an Illumina iSeq 100 (2 x 150bp).

399 *Genome Assembly and Analysis*

400 All sequencing reads were demultiplexed using the Illumina BaseSpace Sequence Hub. The ancestral
401 genome was first mapped to the *S. marcescens* KZ19 (GCA_002915435.1) reference genome [25]. In
402 brief, the raw sequencing reads were trimmed using BBDuk (sourceforge.net/projects/bbmap/)

403 implemented in Geneious Prime, using default settings. Trimmed reads were then assembled by
404 mapping to the ancestral genome in Geneious Prime using Bowtie 2 [43] with default settings.
405 Assembled contigs were annotated using RAST [44]. We also mapped all the reads of the ancestral
406 strain back to the consensus ancestral genome using BreSeq [28]. Any mutations predicted by BreSeq
407 when the ancestral reads were mapped to the ancestral consensus genome were considered
408 sequencing or assembly issues in the ancestral genome and removed as candidate mutations in the
409 evolved populations and isolates.

410 The sequencing reads of the evolved populations and isolates were trimmed using Trimmomatic
411 (46). The trimmed reads were then mapped to the ancestral genome using BreSeq version 0.37.0 [28]
412 with a base-quality-cutoff PHRED score of 30 to identify mutations. Promoters were predicted using
413 iProEP [45]. Functional characterization of genes (Figure 2- Table Supplement 2) was predicted using
414 KOALA KEGG Orthology And Links Annotation (48). Simulations to determine the percent mutations
415 expected at random across intergenic, synonymous and non-synonymous positions were conducted
416 with a custom Python script: we conducted 10,000 independent simulations where one mutation was
417 introduced at random under a Jukes & Cantor model in the refence genome of *S. marcescens* KZ19
418 (GCA_002915435.1). The annotations of the genome were used to infer whether the mutation was
419 intergenic or not. Mutations affecting a coding sequence were inferred as synonymous or non-
420 synonymous by translating the gene *in silico* before and after introducing the mutation.

421 *Materials availability*

422 All raw sequencing data has been deposited on NCBI under BioProject PRJNA838621.

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429 **Author Contributions**

430 H.A.H. performed the research, K.R. designed research, H.A.H. and K.R. analyzed data and wrote the
431 paper.

432 **Competing Interest Statement**

433 The authors declare no completing interests.

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545

546 **Supporting Material**

547

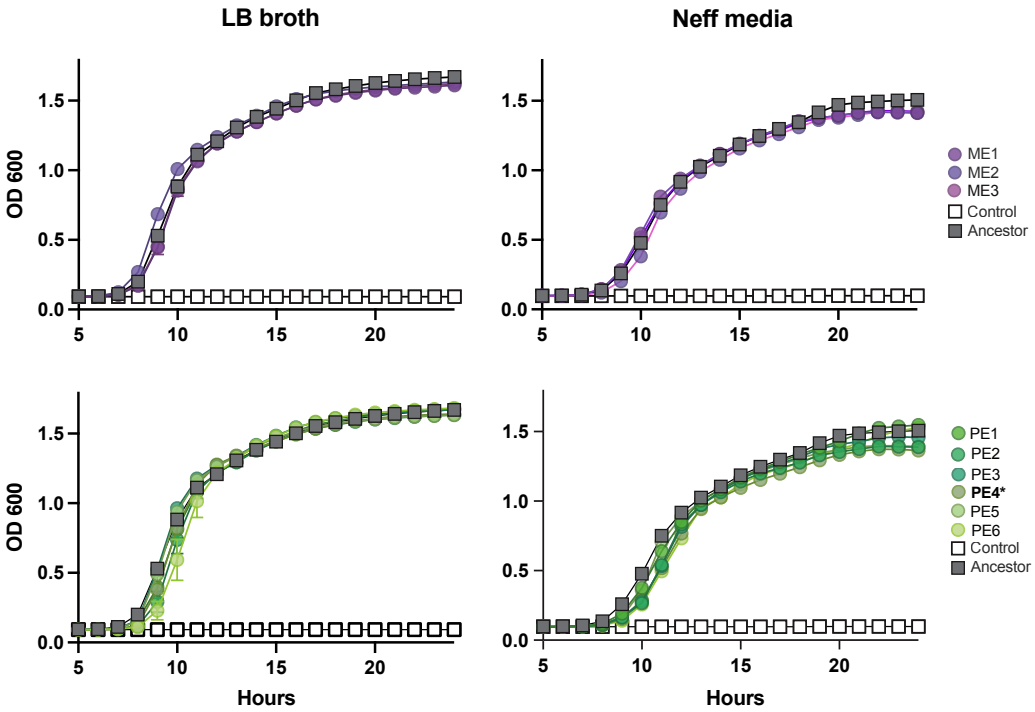

Figure S1. Mean growth rate of each variant based on 600nm OD readings every hour for 24h of ME (purple) and PE (green) isolates in LB broth (left) or fresh Neff media (right) compared to the ancestral strain (black).

Table S1. Total reads, number of mapped reads, and average genome coverage for each population and isolate.

Table S2. List of parallel mutations identified across evolved populations.

Table S3. List of all the mutations identified in the evolved isolates and their frequency in the populations.

Dataset S1. List of all the mutations identified in the evolved populations and their frequencies.