Opportunistic pathogen virulence is maintained by the presence of predators

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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, 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 or increased 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 strains revealed multiple cases of parallel evolution in genes associated with virulence. Our results provide evidence that bacterial virulence is 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. The ability of opportunistic pathogens to occasionally infect hosts is 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 that drive 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 is the result of exaptation, i.e., virulence evolves indirectly due to selection that occurs in non-host environments (3, 4). Specifically, bacterial defense mechanisms against environmental predators (e.g., amoebae, protists, and viruses) 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). Some studies have supported the CEH by showing that pathogens challenged with microbial predators exhibited increased host virulence (7–11), and genes involved in pathogenesis were upregulated during predation (11). In contrast, other studies have demonstrated that exposure to predators can decrease opportunistic pathogens' virulence within hosts (12–16) . Experimental evolution represents a powerful approach for evaluating if predation drives virulence, i.e., evolving a pathogen in the lab in the presence of predators. Yet, of the limited studies that have investigated whether the CEH can explain virulence in opportunistic pathogens, very few have utilized experimental evolution (12–17) and results have been conflicting. Moreover, in most cases, the phenotypic and genotypic changes that resulted

from experimental evolution were not thoroughly examined.

Serratia marcescens is an opportunistic pathogen that ubiquitously occurs in soil and water (18). It is a host generalist that can colonize many different organisms, where it can be pathogenic and/or commensal (18). In humans, *S. marcescens* is a common enteric bacterium that is generally harmless in the gastrointestinal tract (19). However, it was recently found to be capable of damaging gut epithelial cells *in vitro* (20) and is responsible for causing dangerous nosocomial infections worldwide, notably in the respiratory and urinary tracts (21, 22). 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 (21, 22). Additionally, *S. marcescens* has been recognized as an opportunistic pathogens of many plants and other animals (18). Because *S. marcescens* is a host generalist, can cause diverse types of infections, and has multidrug resistance, understanding what factors contribute to the evolution of its virulence is important for the health of numerous organisms. Furthermore, studying the evolution of virulence in *S. marcescens* can shed light on the processes driving virulence in other opportunistic pathogens.

Here, we evolved *S. marcescens* in the presence or absence of a ubiquitous environmental protist predator, *Tetrahymena thermophila*. We sequenced the genomes of isolates (strains) from multiple evolved lines and characterized the mutations that occurred during evolution. We tested the fitness of the evolved strains using biofilm, growth, and predation resistance assays. We performed survival assays to test the virulence of the evolved strains in a natural animal host (23), the honey bee (*Apis mellifera*). Overall, our results reveal that virulence is attenuated in the absence of a predator, even over short evolutionary timescales, and demonstrate that predation plays a key role in maintaining virulence in opportunistic pathogens. We found that adaptation to both a predator and predator-free environment often resulted in fitness tradeoffs. Additionally, we observed multiple cases of parallel evolution in genes that have been associated with pathogenicity. In conclusion, we provide strong evidence that virulence is maintained by selective pressures imposed by predators, supporting the CEH.

Results

We evolved *S. marcescens* KZ19, isolated from honey bees (23), by serially passaging every day for 60 days (in Neff media (24)) in the presence or absence of a ubiquitous generalist protist predator *T. thermophila* (Figure 1). In our study we controlled for predator/prey co-evolution by only experimentally evolving *S. marcescens*, i.e., evolved bacteria were passaged to new *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 also evaluated before every passage via light microscopy. Following 60 days of evolution we randomly isolated 2-5 colonies per line (Figure 1). We sequenced the genomes and characterized the virulence and fitness of 30 evolved isolates (10 ME and 20 PE) as well as the ancestral strain.

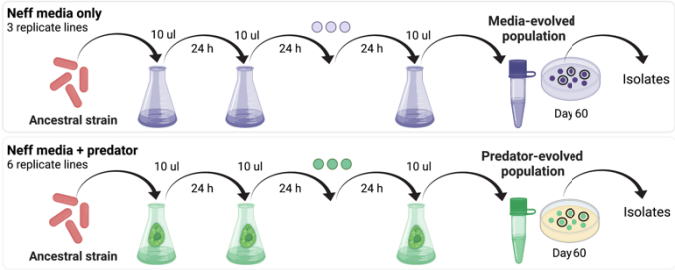

Figure 1. Experimental design and number of isolates analyzed per evolved population (line). Isolates used in this study were named based on the line from which they were isolated (e.g., ME1-1 = Media-Evolved Line 1 Isolate 1).

Mutations in the predator- and media-evolved strains

The genomes of the 10 ME, 20 PE, and the ancestral strain were sequenced using WGS sequencing. The average genome coverage was 58X (Table S1). All evolved 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. We then corrected for false positives by mapping all the

reads of the ancestral strain back to the new consensus ancestral genome using BreSeq (25). All mutations predicted via BreSeq (25) when the ancestral reads were mapped to the ancestral consensus genome were not considered real mutations if detected in the evolved isolates (i.e., they most likely correspond to sequencing or assembly issues in the ancestral genome). At least one mutation was detected in all evolved isolates, with a maximum of six and an average of three mutations per isolate (Table S2). Specifically, we identified non-synonymous, synonymous, and intergenic mutations (i.e., insertions, deletions, and single nucleotide variants (SNVs)) within or upstream (in promoters) of 28 different genes. Some strains isolated on Day 60 were clones, i.e., genetically identical, and were grouped together resulting in at total of six ME and 12 PE unique variants (Table 1, Figure S1). Clones were mostly identified within the same evolved population, but we did observe one case where two isolates from different PE lines were identical (PE 3-1 and PE 4-1).

Variant	Evolved Line(s)	Isolate Name(s)	Color Code
ME1	ME Line 1	ME1-1, ME1-2, ME1-3	
ME2	ME Line 2	ME2-1	
ME3	ME Line 2	ME2-2	
ME4	ME Line 2	ME2-3	
ME5	ME Line 3	ME3-1, ME3-3, ME3-4	
ME6	ME Line 3	ME3-2	
PE1	PE Line1	PE1-1, PE1-2	
PE2	PE Line1	PE1-3	
PE3	PE Line 2	PE2-1, PE2-2, PE2-4	
PE4	PE Line 2	PE2-3	
PE5	PE Line 2	PE2-5	
PE6	PE Line 3, PE Line 4	PE3-1, PE4-1	
PE7	PE Line 3	PE3-2	
PE8	PE Line 4	PE4-2	
PE9	PE Line 5	PE5-1, PE5-2	
PE10	PE Line 5	PE 5-3	
PE11	PE Line 6	PE6-1, PE6-2, PE6-3, PE6-4	
PE12	PE Line 6	PE 6-5	

Table 1. List of unique variants indicating the evolved line they were isolated from, the original isolate name, and the color code assigned to each variant.

Disregarding identical clones or mutations that occurred in isolates from the same evolved line, we found 23 non-synonymous, six synonymous, and eight intergenic mutations across our ME and PE variants (Figure 2A). Of the 22 genes in which non-synonymous and synonymous mutations occurred, 77% could be functionally classified (Figure 2B, Table S3). The 17 characterized genes belonged to six functional classes: metabolism (22.7%), genetic information processing (18.2%), environmental information processing (18.2%), cellular processes (4.5%), organismal systems (4.5%), human diseases (4.5%). Several genes in which we observed mutations have been associated with virulence factors in *S. marcescens* or related enterobacteria, such as *yfiN* (26, 27), *rcsB* (28, 29), and *barA* and its response regulator *uvrY* (30–32). Note that in ME1 a single 5,601 bp deletion affected six genes annotated as 1) Maltose/glucose PTS system component malX, 2) Mal regulon transcriptional regulator malI, 3) LysR family transcriptional regulator, 4) ampC beta-lactamase class C, 5) LysR family transcriptional regulator, and 6) serine hydrolase. Additionally, all intergenic mutations identified are located within promoters upstream of six different genes (Figure 2A).

Remarkably, we identified three instances of site-specific parallel evolution (mutations in the *exact* same position in isolates from different lines) and three cases of gene-specific parallel evolution (mutations in the same gene in isolates from different lines). The most striking case of site-specific parallel evolution was observed in ME strains, where the same 66 bp in-frame deletion was found in the response regulator *rcsB* (33) in all isolates from ME Lines 1 and 3 (variants ME1, ME5 and ME6). The two other site-specific parallel evolution events occurred in promoters upstream of a methyl-accepting chemotaxis sensor/transducer protein in all isolates from ME Lines 2 and 3 (variants ME2, ME3, ME4, ME5, and ME6) and upstream of an uncharacterized hypothetical protein in all isolates from PE Lines 3 and 4 (variants PE6, PE7, and PE8) (Figure 2A). Two of the gene-specific parallel evolution events, which included non-synonymous SNVs, deletions, and one insertion, arose in both PE and ME variants from different lines, and impacted two genes annotated as *barA* (a signal transduction histidine-protein kinase) and *uvrY* (the BarA-associated response regulator) (Figure 1A). The third case of gene-specific parallel evolution arose in a gene annotated as the inner membrane protein *yfiN* in eight variants from four different PE lines (Figure 2A). Cases of parallel evolution found across PE and ME lines suggest adaptation to the media, whereas parallel evolution events unique to either PE or ME lines suggest adaptation to the presence or to the absence of predation, respectively.

We investigated what type of selective pressures (positive, neutral, or purifying selection) the identified mutated genes were under by comparing them to all other genes in *S. marcescens*

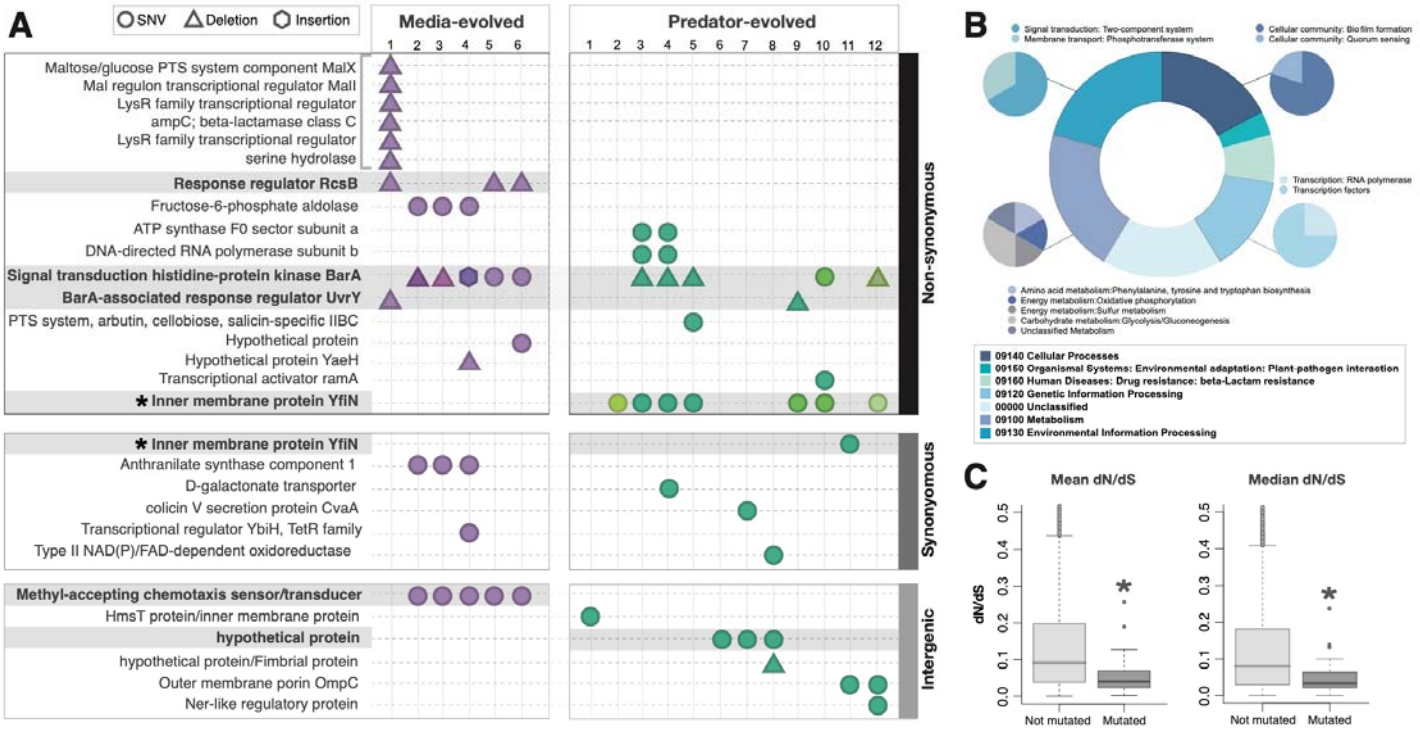

Figure 2. Mutations in the predator- and media-evolved variants**. A)** Mutations identified in the predator-evolved (PE 1-12) and media-evolved (ME 1-6) variant genomes. Mutations included insertions (hexagons), deletions (triangles), and single nucleotide variants (circles), and were non-synonymous (black vertical bar), synonymous (dark gray vertical bar), and intergenic (light gray vertical bar) mutations. Shapes with the same color or shade within the same row represent mutations that occurred in the exact same position. Shapes with different color or shade within the same row represent mutations that occurred in different locations within the same gene. Light gray highlighting of a row indicates cases of parallel evolution across evolved lines. **B)** Relative abundance pie graphs showing the KEGG functional annotations of the genes that contained non-synonymous and synonymous mutations (see Table S3 for full annotation information). **C)** Mean and median *dN/dS* ratio of the genes in which we identified mutations versus all other genes in the pan-genome of *S. marcescens* (484 genomes). Evolved isolates were not included in the dN/dS analysis. ***** = P<0.01; Wilcoxon Sign Rank Test.

Even though most of the mutations that we identified were non-synonymous, we found that the genes in which we identified mutations are more constrained by purifying selection (mean and median *P*<0.01; Wilcoxon Sign Rank Test) than all other genes in the pan-genome of *S. marcescens* (Figure 2C). This finding implies that either the mutations we identified have not yet been purged from the population or are beneficial under our experimental conditions.

Virulence of predator- and media-evolved strains

S. marcescens KZ19 is pathogenic in honey bees under certain circumstances, e.g., following antibiotic treatment, when exposed to high doses, or when injected into the hemolymph (23). Thus, we 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 20 predator-evolved (from 6 different lines) and 10 media-evolved (from 3 different lines) isolates by comparing them to the virulence of the ancestral strain. Three assays were done with five replicates each, totaling 300 bees per isolate (Figure 3, Figure S2). It is important to note that all virulence and fitness assays described below were performed prior to genomic sequencing. Therefore, all isolates were initially analyzed separately (Figures S2-S5) but after sequencing the identified clones were combined to generate Figure 3 and for statistical testing.

Nearly all the ME variants displayed attenuated virulence when compared to the ancestral strain, with half being significantly less virulent (Figure 3; *P*<0.01, Mantel-Cox Log-rank test with Bonferroni correction). Conversely, most of the PE variants appeared to be more virulent than the ancestor but only one (PE7) was statistically

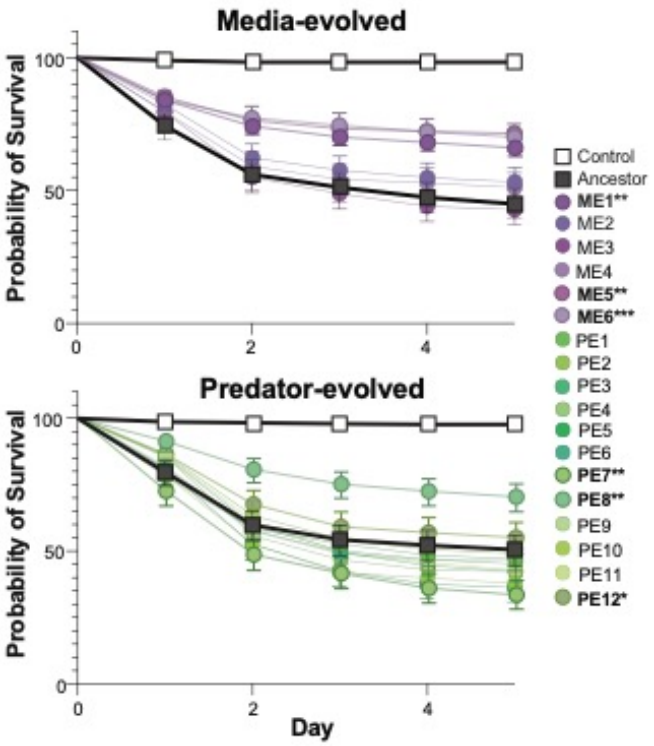

Figure 3. Probability of survival of honey bees after high dose exposure to the ancestor (black squares), ME variants (left, purple circles), and PE variants (right, green circles). Control bees were given sterile sugar water only (white squares). Mortality was recorded each day for 5 days. Graphs show mean and standard deviation of the probability of survival based on three replicate assays per isolate (see Figure S2 for individual assays). Note that some variants contain multiple isolates (Table 1). Survival curves were created in GraphPad Prism. Statistical testing was performed using the Mantel-Cox Log-rank test with Bonferroni correction. * = P<0.01, ** = P<0.001, and *** = P<0.0001

significant (Figure 3; *P*<0.01, Mantel-Cox Log-rank test with Bonferroni correction). Two variants (PE8 and PE12) were significantly less virulent than the ancestor (Figure 3; *P*<0.01, Mantel-Cox Log-rank test with Bonferroni correction). Overall, results show that in the absence of predation, virulence is attenuated, and predation maintains or increases virulence in *S. marcescens* (Figure 3, Figure S2). Findings also indicate that some of the PE isolates followed different evolutionary trajectories during the 60 days of evolution.

Fitness of predator- and media-evolved strains

To test the fitness of the PE and ME strains, we performed growth, biofilm formation, and predation resistance (population size after growth in the presence of *T. thermophila*) assays. For growth rate, we conducted *in vitro* assays using three different media types A) fresh Neff, B) spent Neff (filtered media that had supported *T. thermophila* growth for 24h), and C) LB broth. In all media types, virtually all the ME and PE variants displayed a slower growth rate than the ancestor over 24h, albeit not substantially so (Figure 4, Figure S3). In general, growth rate of the PE variants was more attenuated than the ME variants (Figure 4, Figure S3). This pattern suggests a tradeoff between growth rate and resistance to predation.

Because biofilm formation is a strategy for resistance to predation and a known virulence factor, we measured biofilm production in our ME and PE variants compared to the ancestor (Figure 4D, Figure S4). Overall, the ME variants did not differ (*P*=0.28, Mann Whitney test with Bonferroni correction) from the ancestor in terms of biofilm production, whereas the PE variants produced significantly more biofilms than the ancestor (*P*<0.001, Mann Whitney test with Bonferroni correction). In fact, biofilm production increased between 1.4- to 2.6-fold across PE variants relative to the ancestor, indicating that they are producing more biofilms due to predation.

Lastly, we evaluated predation resistance (i.e., growth with predator) of the ME and PE variants. Each isolate was grown with *T. thermophila* for 48h and then the population density of the bacteria was measured (Figure 4E, Figure S5). In general, predation resistance of the PE variants did not significantly differ from that of the ancestor (*P*>0.01; Wilcoxon Signed Rank Test with Bonferroni correction). However, predation resistance across PE variants differed with some being similar to the ancestor, whereas others appeared to be more resistant or less resistant to predation relative to the ancestor (Figure 3E). Overall, the ME isolates were significantly less resistant to predation than the ancestor (*P*<0.001; Wilcoxon Signed Rank Test with Bonferroni correction). Only two ME variants (ME3 and ME4) did not noticeably decrease in population size after co-culture with *T. thermophila* when compared to the ancestor (Figure 4E). Results suggest that lack of predation decreases grazing resistance while predation maintains it. Findings further confirm that the PE lines followed different evolutionary paths over the 60 days of evolution.

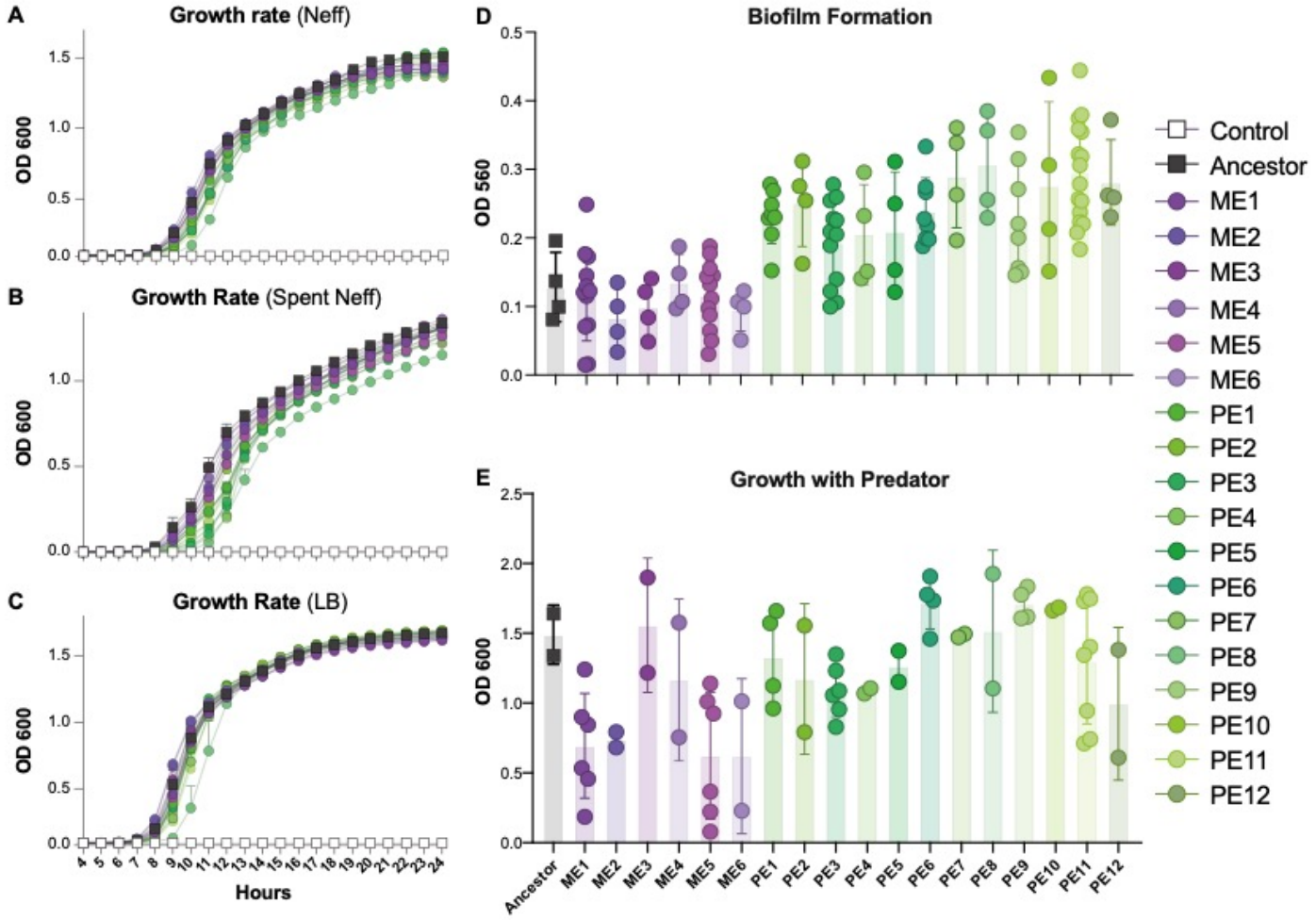

Figure 4. Fitness of predator- and media-evolved variants. **A-C)** Mean growth rate based on 600nm OD readings every hour for 24 hours of PE (green) and ME (purple) variants in **A)** fresh Neff media **B)** spent Neff media and **C)** LB broth compared to the ancestral strain (black). **D)** Mean biofilm formation (from four replicate trials; Figure S4) of PE variants, ME variants, and the ancestral strain based on 560nm OD readings after 36 hours of growth. Bars represent the mean across trials, points represent the mean for each isolate per variant from the four replicate trials (note: some variants contain multiple isolates), and the error bars show the standard deviation. Biofilm production increased in the PE variants when compared to the ancestor ($P<0.001$, Mann Whitney test with Bonferroni correction). ME variants did not significantly differ from the ancestor in biofilm production ($P=0.28$, Mann Whitney test with Bonferroni correction). **E)** Mean predation resistance (from two replicate trials; Figure S5) of PE variants, ME variants, and the ancestral strain based on 600nm OD readings of bacterial filtrate after 48 hours in co-culture with *T. thermophila*. Bars represent the mean across trials, points represent the mean for each isolate per variant from the two replicate trials (note: some variants contain multiple isolates), and the error bars show the standard deviation. Predation resistance did not differ between the ancestor and the PE isolates ($p>0.01$; Wilcoxon Signed Rank Test with Bonferroni correction). ME isolates were significantly more resistant to predation than the ancestor ($p<0.001$; Wilcoxon Signed Rank Test with Bonferroni correction).

Discussion

Evolution of *S. marcescens* in the absence of a predator resulted in attenuated virulence in most of the variants investigated when compared to the ancestral strain. Conversely, *S. marcescens* that were evolved in the presence of a predator showed a general trend of increased virulence, albeit only one was significantly more virulent and two were significantly less virulent than the ancestor. The differences observed across evolved isolates are likely the result of different lines/isolates undergoing alternative evolutionary paths which did not always confer increased or decreased virulence during the course of our 60-day experiment. Nevertheless, our results indicate that virulence is quickly lost in the absence of predation but is largely maintained in the presence of

predation. Maintenance rather than increased virulence following evolution with a predator could be due to the fact that the ancestral strain (KZ19) is a natural opportunistic pathogen of honey bees (23) and may have limited potential to become more virulent, especially in only 60 days of evolution and because of the protection provided by the native gut microbes (23, 35).

We found that the growth rate of PE and ME variants did not substantially differ from the ancestor. Nevertheless, growth rate of the PE variants was systematically lower than the ME variants, likely revealing fitness tradeoffs involved in adaptation to predation. Biofilm formation was increased in all the PE variants when compared to the ancestor and the ME variants. Biofilm production is a natural defense mechanism against predation and has been shown to play a role in pathogenicity (36). Our results revealed that ME variants were significantly less fit in the presence of a predator than the ancestor while the PE variants maintained a similar population size as the ancestor, suggesting evolution in the presence of a predator sustains resistance to predation. Additionally, the ME variants that displayed attenuated virulence were also the most susceptible to predation (i.e., had to smallest population size after co-culture with *T. thermophila*), implying that virulence and predation resistance are governed by the same mechanisms. It is important to note that we observed phenotypic differences after only 60 days of evolution and with only one to six identified mutations per variant.

Several different mutations, including some in genes associated with virulence, arose in the ME and PE variants. Most mutations observed were non-synonymous or located in promoters, but synonymous mutations were also identified. Despite being mostly affected by non-synonymous mutations, the genes in which we identified mutations were found to be under stronger purifying selection than all other genes in the pan-genome of *S. marcescens*. Thus, they have either not yet been purged from the population or are beneficial in our experimental scenarios. Consistent with the latter hypothesis, multiple cases of site-specific and gene-specific parallel evolution were observed, indicating that some genes are under strong selection in the presence and/or absence of a predator. Most instances of parallel evolution involved non-synonymous or intergenic mutations within promoters, both of which likely impact gene expression and/or function. However, one case of parallel evolution also included mutations that were synonymous. Though generally thought to be “silent”, recent studies have revealed that synonymous mutations can have an impact on gene expression (37, 38). Therefore, the synonymous mutations identified in our evolved isolates could have an impact on fitness, especially those that were observed across multiple evolved populations.

Gene-specific parallel evolution events occurred in two genes annotated as *barA* and *uvrY* in most of our ME and PE variants. In *Escherichia coli*, *barA* and *uvrY* encode a two-component system which plays an important role in the ability to switch between different carbon sources (30). Additionally, orthologs of BarA and UvrY have been associated with virulence in several Gram-negative pathogens (30–32). Some of the mutations that we observed most likely resulted in a loss of function of one or both genes since they involved out-of-frame deletions. In other variants, it is possible that the mutations altered the function or expression of *barA* and/or *uvrY* since they consisted of a SNV or in-frame deletions of 6-9bp. Based on the evidence that the BarA-UvrY controls the carbon storage regulatory system in *E. coli* (30) and that mutations in these genes arose in both PE and ME variants, we predict that they are the result of adaptation to the Neff media, which consists of proteose peptone, yeast extract, and glucose.

We also identified cases of parallel evolution that were specific to either ME or PE variants, signifying that they are likely due to adaptation to the presence or to the absence of a predator rather than the media. For example, in all isolates from ME Lines 1 and 3 (variants ME1, ME5, and ME6), the same 66bp in-frame deletion (site-specific parallel evolution) occurred in a gene annotated as *rcsB*. RcsB is the response regulator of the Rcs phosphorelay two-component signal transduction system in enterobacteria (28). RcsB has been associated with the synthesis of biofilms, capsules, and flagella and antibiotic resistance (28). Isolates that contained the deletion in *rcsB* displayed significantly attenuated virulence and had much lower population sizes after co-culture with *T. thermophila* than the ancestral strain, indicating that

RcsB plays a role in predation resistance and pathogenicity in *S. marcescens*. One other case of site-specific parallel evolution found in most ME variants was an intergenic mutation in the promoter upstream of a methyl-accepting chemotaxis sensor/transducer protein. Methyl-accepting chemotaxis proteins are used to sense and respond to environmental stimuli and are involved in the synthesis of biofilms, flagella, and toxins in many pathogens (39). The fact that this mutation was in a promoter and found in multiple isolates from different evolved lines suggests that it confers a fitness benefit in the absence of predators.

In most of the PE variants, both non-synonymous and synonymous SNVs were found in a gene encoding the inner membrane protein YfiN (gene-specific parallel evolution). YfiN has been implicated in biofilm formation, inhibiting motility, and cell division arrest under stressful conditions (26, 27). The fact that mutations arose in *yfiN* independently across different PE lines implies that this gene is under strong selective pressure in the presence of predators. Mutations in *yfiN* were identified in eight of the 12 PE variants and we speculate that they are responsible for the observed increase in biofilm production. The PE variants that did not acquire a mutation in *yfiN* either contained a site-specific parallel evolution event in the promoter upstream of a hypothetical protein (unknown function) or the promoter upstream of the inner membrane protein HmsT. HmsT is associated with biofilm formation in *Yersinia pestis* (40), the closest known relative of *S. marcescens*. Although only a speculation, we predict that the intergenic mutations in the promoter regions upstream of HmsT and the hypothetical protein are involved in increasing biofilm formation in the PE variants that did not contain mutations in *yfiN*.

Of the limited number of previous experimental evolution studies that directly tested the applicability of the CEH for opportunistic pathogens, most concluded that predation decreases virulence (12–16), despite the fact that many also used *S. marcescens* as their model system (12, 13, 15). However, virulence and other phenotypes were evaluated either based on testing i) a mixture or a population of evolved isolates (12, 16) or ii) individual isolates that were then combined to generate the results and/or using different isolates for various assays (13–15). Both scenarios are problematic: By testing a mixture of evolved isolates, it is likely that one will outcompete the others (and that the “winner” will be different depending on the environment). Combining or comparing data from assays performed with different isolates could strongly impact the results since each isolate might genotypically and phenotypically differ (as we observed here) and be more or less fit depending on the context. To our knowledge, only one previous study sequenced the genomes of evolved isolates or populations following experimental evolution with and without a predator, but the genotypes of isolates used for phenotypic characterization were not reported (16). Moreover, the authors claim to have identified over 400 mutations in their evolved isolates after only three days (16), which even in the absence of natural selection seems highly unrealistic. Indeed, mutation accumulation (MA) 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 even in the absence of natural selection (41). The excessive number of mutations reported could be due to contamination (not starting with a single clone) and/or because the authors reported mutations even if they were only represented by 10% of the mapped reads, increasing the chance of confounding sequencing errors with mutations. Comparatively, in the Long-Term Evolution Experiment (LTEE) only 42 mutations occurred in an entire evolved *E. coli* population over the course of 20,000 generations (approximately 3,000 days) (42, 43) and after 60 days, some of our isolates only contained a single mutation. Therefore, we argue that the inconsistency between our findings and similar predator/prey experimental evolution studies is likely the result of using inadequate methods to assess phenotypes and genotypes.

One caveat of our study is the fact that we performed all virulence and fitness assays on each random isolate (10 ME and 20 PE) before performing genomic sequencing. After sequencing we identified that some of our isolates were genetically identical (clones). Thus, to avoid pseudo-replication we had to combine the clones post hoc for statistical analysis, resulting in some variants containing more data than others. However, combining clones did not alter the general results or conclusions of our study and we included all assays performed on each individual isolate in the

supplemental material for transparency (Figures S1-S5). Another limitation we would like to acknowledge regards our assumption that *T. thermophila* are actively preying on (consuming) *S. marcescens*. We did not specifically investigate if and how much the *T. thermophila* consumed *S. marcescens* so it is possible that the genotypic and phenotypic changes we observed were due to competition for resources rather than predation. Though, we have strong indirect evidence that predation was occurring since most mutations we identified in the PE variants occurred in genes involved in biofilm formation, we observed increased biofilm production in these variants, and biofilm formation is a natural defense mechanism against predation. Moreover, it seems highly unlikely that increased biofilm formation would provide an advantage to compete for resource acquisition. Regardless of the exact mechanism (competition or predation) driving the evolution of *S. marcescens* in the presence of a predator, our results still confirm that selective pressures imposed by a predator play a role in maintaining virulence.

In conclusion, our results suggest that predation helps maintain virulence in opportunistic pathogens, thereby supporting the CEH. We identified several instances of parallel evolution across evolved lines, which, when combined with our phenotypic assays, allowed us to infer the outcome of some mutations. For example, we found evidence that BarA and UrvY are involved in carbon utilization in *S. marcescens*. We also predict that the mutations we observed in *yfiN* and two promoters (upstream of HmsT and of a hypothetical protein) are responsible for increased biofilm production. Moreover, our results imply that RscB plays a major role in predation resistance and virulence in *S. marcescens*. Fundamentally, our study demonstrates the efficacy of using experimental evolution to identify virulence mechanisms, even over short evolutionary timescales. Performing long-term evolutionary experiments will provide a more accurate picture of the forces that drive the evolution of opportunistic pathogens and the mechanisms responsible for their virulence. By identifying virulence mechanisms in opportunistic pathogens, new treatment strategies for combating multidrug resistant pathogen infections can be developed.

Materials and Methods

Experimental evolution

Serratia marcescens strain KZ19 was evolved either in media alone (media-evolved) or with the predator *Tetrahymena thermophila* strain SB210 (predator-evolved). *T. thermophila* strain SB210 was purchased from the Tetrayhema Stock Center located at Cornell University. The media-evolved (ME) experiment included three replicate lines and the predator-evolved (PE) experiment included six replicate lines. Each replicate line was started by adding 10µL of 10^4 bacterial cells/mL to either 3mL of Neff media (24) alone (ME lines) or 3mL Neff media containing equal concentrations of *T. thermophila* (PE lines). Cultures were incubated at 30°C without shaking. Every 24h for 60 days, 10µL of each culture was sampled from the flasks and added to 3mL of either 1) fresh Neff media or 2) new axenic cultures of *T. thermophila* (to avoid co-evolution) in Neff media. Every day, prior to passaging, the cultures were checked for *T. thermophila* viability and density via microscopy and *S. marcescens* presence (successful passaging and growth) was evaluated by plating on LB agar. Every day over the course of the 60 day evolution experiment *T. thermophila* and S. marcescens were always detected and we never observed a noticeable difference in population size or biofilm formation.

On day 60, six Luria-Bertani (LB) agar plates were streak inoculated from the culture flasks and incubated for 24h at 30°C. Twenty bacterial colonies were randomly selected from the PE lines and ten bacterial colonies were randomly selected from the ME lines (Figure 1). Pure cultures of each isolate (30 total) were created by inoculating in 3mL LB broth with 24h incubation at 30°C. Samples (200µL from each pure culture) were then frozen at -80°C in 20% glycerol (*T. thermophila* do not survive freezing). These 30 isolates were used for sequencing and all phenotypic analysis.

Virulence assays

Pure cultures of the evolved isolates and the ancestor (31 total) were diluted to 0.5 OD in a 1:1 sterile sugar syrup solution (SSS). Conventional adult honey bee workers were sampled from a single hive and exposed via the immersion method (23) to ~10µL of one of three treatments: 1) sterile SSS only, 2) the ancestor in sterile SSS, or 3) each evolved isolate in sterile SSS. Bees were kept in

cup cages (20 bees/cup; 100 bees/treatment) under hive conditions: 35°C and 95% humidity. Mortality between the treatments was noted every day for 5 days. Assays were performed in triplicate (300 bees tested in total per isolate). Individual survival graphs were created for each individual assay performed (Figure S2). All replicate virulence assays were then combined to generate a single survival curve for each evolved variant (Figure 3). Survival curves (Kaplan-Meier), and statistical analyses (Mantel-Cox Log-rank tests) were done using GraphPad Prism version 9.1.0. For statistical analyses the data from clones and all replicate trails were combined before comparing to the ancestor in order to account for pseudo-replication, variation across trials, and to avoid multiple comparisons with few data points.

Fitness assays

Growth assays were performed in triplicate in both LB broth, fresh Neff media, and spent Neff media with *T. thermophila* filtered out (i.e., *T. thermophila* were present in the media for 24h before filtering). Pure cultures of the evolved strains and the ancestor were diluted to 10^4 bacterial cells/mL, with 5µL of the culture pipetted into 200µL of LB broth of a 96-well microplate. Absorbance detection was performed at an OD of 600nm at 30°C with shaking every hour for 24h on a BioTek Synergy™ 2 plate reader. Growth curves were created using GraphPad Prism version 9.1.0. All isolates were analyzed individually (Figures S3) and identical clones were combined post hoc to generate Figure 4A-C.

Biofilm assays were repeated four times in triplicate for each isolate. Pure cultures of the evolved strains and the ancestor were diluted to 10^4 bacterial cells/mL, with 5µL of the culture pipetted into 200µL of LB broth of a 96-well microplate. The covered plate was incubated for 36h at 30°C without shaking. After 36 hours, the supernatant was poured off, the wells were washed twice with 200µL of sterile ddH2O, and the cells were stained with 150µL of 0.04% crystal violet for 10 minutes. After 10 minutes, the excess crystal violet was removed, the wells were washed again and stained once more for 10 minutes. The crystal violet was poured off and cells were solubilized with 150µL of 95% ethanol. OD readings were taken at absorbance reading 560nm on the BioTek Synergy™ 2 plate reader. All graphs and statistical analyses (Mann Whitney tests) were performed in GraphPad Prism version 9.1.0. For statistical analyses the results from all ME or PE variants from all replicate trials were combined into a ME or PE dataset before comparing to the ancestor in order to account for variation across trials and avoid multiple comparisons with few data points.

Predation resistance assays were performed three times in triplicate for each isolate. Pure cultures of the evolved strains and the ancestor were diluted to 10^5 bacterial cells/mL, and 1mL was added to a culture flask containing *T. thermophila* in 3mL Neff media. *T. thermophila* alone in 3mL Neff media served as the negative control. Experimental culture flasks were incubated at 30°C for 48h. After 48h, the cultures were mixed thoroughly and 1mL was sampled and centrifuged at 5,000 rpm for 10 minutes. The supernatant was discarded, pellets were resuspended in 1.5mL 1X PBS, and the solution was filtered through 5.0 µm MilliporeSigma™ Millex™-SV Sterile PVDF syringe filters. OD readings were taken of filtrates at 600nm after blanking with the negative control filtrate. All graphs and statistical analyses (Mann Whitney tests) were performed in GraphPad Prism version 9.1.0. For statistical analyses the results from all ME or PE variants from all replicate trials were combined into a ME or PE dataset before comparing to the ancestor in order to account for variation across trials and avoid multiple comparisons with few data points.

DNA Extraction and Sequencing

Pure cultures of the 30 evolved isolates and the ancestor were diluted to 1 OD. DNA extractions of the evolved strains were performed using the Zymo Quick-DNA Fungal/Bacterial Miniprep Kit (D6005). The DNA was then prepared for Whole Genome Shotgun (WGS) sequencing using the Illumina Nextera DNA Flex Library Prep Kit (20018704). Genomes were sequenced in-house on an Illumina iSeq 100 (2 x 150bp).

Genome Assembly and Analysis

All sequencing reads were demultiplexed using the Illumina BaseSpace Sequence Hub. The ancestral genome was first mapped to the *S. marcescens* KZ19 reference genome (23). In brief, the raw sequencing reads were trimmed using BBDuk (sourceforge.net/projects/bbmap/) implemented in

Geneious Prime, using default settings. Trimmed reads were then assembled by mapping to the ancestral genome in Geneious Prime using Bowtie 2 (44) with default settings. Assembled contigs were annotated using RAST (45).

The sequencing reads of the evolved isolate were trimmed using Trimmomatic (46). The trimmed reads were then mapped to the ancestral genome using BreSeq (25) to identify mutations in the evolved isolate genomes (Table S2). To confirm that the mutations were not artifacts corresponding to sequencing or assembly issues in the ancestral genome, we also mapped all the reads of the ancestral strain back to the consensus ancestral genome using BreSeq (25). All mutations predicted via BreSeq (25) when the ancestral reads were mapped to the ancestral consensus genome were considered false positives. The average genome coverage for all strains was between 25X-118X (Table S1). Our threshold for calling a SNP was that it had to be represented by 90% or more of the mapped reads. Promoters were predicted using iProEP (47). Functional characterization of genes was predicted using KOALA (KEGG Orthology And Links Annotation) (48).

We calculated *dN/dS* ratios for each gene in the pan-genome of 484 *S. marcescens* genomes (used in (34) and available on NCBI). Protein sequences were aligned with MUSCLE v3.8 (49) and reverse translated into their corresponding nucleotide sequences. Pairwise *dN/dS* ratios were computed with PAML v4.9 (50) using the yn00 algorithm. For each gene alignment, the average *dN/dS* ratio was reported across all pairwise comparisons. Graphs and statistical analysis conducted in R (51).

Data availability

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

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Author Contributions

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

Competing Interest Statement

The authors declare no completing interests.

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