EVOLUTION OF INDEPENDENT GENETIC PATHWAYS FOR PATHOGEN RESISTANCE WITHIN THE NEMATODE *CAENORHABDITIS REMANEI*

by

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THESIS ABSTRACT

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Pathogenic host-microbe interactions can result from continuous evolution of a host's ability to resist infection and a pathogen's ability to survive and replicate. *Pseudomonas aeruginosa* is a versatile and opportunistic pathogen, ubiquitous in soil, and capable of damaging plants, vertebrates, and invertebrates. Previous studies in nematodes suggest that the pathogenic effects of *P. aeruginosa* can result from multiple distinct pathways: a toxin-based effect that kills within a few hours and a generalized virulence that kills over the course of multiple days. Using experimental evolution in the highly polymorphic nematode *Caenorhabditis remanei*, I show that nematode resistance to the two modes of pathogenesis in *P. aeruginosa* evolves through genetically independent pathways. These results demonstrate that multiple virulence factors in a pathogen can result in multiple responses in the host, and the genetic lines established here create resources for further exploration of the genetic basis for resistance to *P. aeruginosa*.

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Dedicated to Trudy Scott Bradley, 1948 – 2012. The infinite possibilities each day holds should stagger the mind. The sheer number of experiences available is uncountable, breathtaking. Yet so many live trapped in loops. Reliving the same days over and over, envisioning only a handful of paths that have been laid out ahead. Each day seeing the same things, responding the same way, a slight variation of yesterday. Every moment smoothly following the gentle curves of norms and expectations. Thinking that tomorrow our dreams can become reality (modified from XKCD: Dreams).

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CHAPTER I

INTRODUCTION

Pathogenic host-microbe interactions can be likened to an ongoing battle, a continuous evolution between host ability to restrict infecting microbes and pathogen ability to survive and replicate. If an important long term goal of health research is to sway the battle in favor of hosts, then it is fundamentally important to understand the genetic mechanisms underlying this exchange. Many insights into host immunity have been made during the past decade, but much of this work has been conducted in cell culture (Baxt, et al., 2013). Studies of this type are somewhat oversimplified due to lack of the contextual and systemic complexity present in vivo. Evidence increasingly shows, for example, that multiple pathogen response pathways exist, interact, and regulate other pathways (Saitoh, et al., 2008; Nakahira, et al., 2011; Irazoqui, et al., 2010). This potential for interaction among otherwise independent pathways underscores a critical need for pathogen-host interaction studies based on intact biological systems that allow analysis of host responses in conditions such that interactions among immune pathways can be observed.

Nematodes provide one such system. These microscopic worms can be free-living or parasitic. Many of the free-living species, such as *Caenorhabditis elegans,* are soil-dwelling bacteriovores. As a model organism, *C. elegans* has been considered ideal due to its small size, transparent body, ability to survive freezing, short generation time, completely mapped cellular differentiation pattern, self-fertilizing reproductive system which can produce genetically identical offspring, ease of targeted genetic disruption via double-stranded RNA-mediated interference (RNAi), and fully sequenced genome with

homology to humans approaching 80% (Kaletta & Hengartner, 2006; Lai, et al., 2000). *C. elegans* is also uniquely tractable for the study of pathogen interactions. In addition to being a bacteriovore, it is susceptible to many of the same pathogens that sicken and kill mammals and humans (O'Callaghan & Vergunst, 2010). Additionally, these worms have interacted with and adapted to bacteria for greater than 600 million years (Irazoqui, et al., 2010). Presumably, during this time bacteria have evolved to avoid predation at least in part via the same mechanisms utilized to avoid detection in humans, while at the same time worms have evolved immune characteristics to overcome pathogenic effects of the bacteria.

Remarkably, many of the genetic elements involved in immune responses were already established when worms and humans last shared a common ancestor nearly one billion years ago (Ruvkun & Hobert, 1998). Several of these components, such as toll-like receptors (TLRs) and anti-microbial peptides (AMPs), have already been shown to be highly conserved across many diverse taxa including insects, mammals, and plants (Ganz & Lehrer, 1994; Broekaert, et al., 1995; Hoffman, 1995; Takeda & Akira, 2003). Moreover, it has also been shown that over the course of evolutionary history that genetic pathways with important contributions to fitness persist and tend to adopt only slightly varied function in related species (De Robertis, 2008). It is plausible then that currently existing components of nematode immunity have been conserved across taxa, including humans, despite the billion years of separation.

For example, studies conducted in *Drosophila melanogaster* first revealed the functional role of Toll receptors as part of a signaling pathway governing immune response with a highly conserved homology to human interleukin 1 ($IL\text{-}_1$) receptors

(Rock, et al., 1998; Wu & Anderson, 1998). In many insect systems the Toll receptor is activated by the cytokine Spaetzle, which is produced in response to detection of pathogen-associated molecular patterns (PAMPs) while vertebrate TLRs are directly activated by PAMPs. However in both cases the downstream pathways are largely the same: TLRs initiate the production of transcription factors belonging to the nuclear factor-κβ (NF-κβ) family through signals transmitted by scaffold proteins of the Mal and Myd88 classes (Akira, et al., 2006). The downstream genes regulated by NF-κβ have been well-characterized to include conserved antimicrobial peptides (AMPs) and pro-inflammatory cytokines (Vallabhapurapu & Karin, 2009). All together this is indicative of a high degree of conservation within the structure and function of immune systems in addition to the sequence homology of individual components.

A reverse genetic analysis of the *C. elegans* genome identified components with similarity to insect and mammalian TLRs and associated signaling pathways (Pujol, et al., 2001). In particular is the presence of TOL-$_1$ (a protein containing a Toll/IL-$_1$R protein-protein interaction, aka TIR, domain), TRF-$_1$ (similar to tumor necrosis factor receptor-associated factor, part of a signal cascade), PIK-$_1$ (IL-$_1$R-associated kinase), and IKB-$_1$ (inhibitor of NF-κβ) (Irazoqui, et al., 2010). Additionally, TIR-$_1$ was identified as a scaffold protein containing a TIR domain homologous to human sterile α- and armadillo-motif-containing protein (SARM), which functions in humans as a negative regulator downstream of TLR_3 and TLR_4 (Carty, et al., 2006). Moreover, despite the lack of any clear homologs of MyD-88 or NF-κβ-like transcription factors (intermediary signaling components in mammalian systems) worms are still capable of producing conserved

AMPs in response to pathogens (Zhu, 2008) indicating they do indeed possess evolutionarily shared immune functions (Irazoqui, et al., 2010).

The high degree of conservation begs the question: how does the immune system evolve to recognize and mount a defense against pathogenic bacteria? In a biological system as complex as humans it is very difficult to study the genetic basis of the immune response, first and foremost due to ethical and logistical issues (e.g. long reproduction times) (Glavis-Bloom, et al., 2012). To address these issues, simpler vertebrate model organisms such as the mouse *Mus musculus* and the zebrafish *Danio rerio* have been widely used (Irazoqui, et al., 2010). But, vertebrates possess evolutionarily novel immune system functions, collectively referred to as the adaptive immune system. However, for an adaptive immune response to occur, evolutionarily conserved, a.k.a. innate, immune functions must first be activated (Song, 2012; Kaisho, et al., 2001; Pasare & Medzhitov, 2004; Iwasaki & Medzhitov, 2004; Akira, et al., 2001; Barton & Medzhitov, 2002; Prinz, et al., 2006). Therefore, understanding the evolution and function of innate immunity is important but is often complicated by crosstalk between the innate and adaptive systems (Parish & O'Neill, 1997; Trinchieri, 1995; Reschner, et al., 2008). A seemingly straightforward solution to this particular issue is the use of invertebrate model organisms as they are lacking adaptive immune components (Irazoqui, et al., 2010). But, compounding this problem is that the natural variety of phenotypes that accompany the high levels of genetic variation in complex systems makes it quite difficult to forecast what the relevant bioactivity will be when observations are based on much simpler organisms with lesser phenotypic variety despite the degree of conservation (Kammenga, et al., 2008; Irazoqui, et al., 2010).

The lack of phenotypes is a particular problem for relating the immune function of *C. elegans* to other biological systems. The obligately selfing hermaphroditic mating system of *C. elegans* has resulted in greatly reduced genetic and phenotypic diversity (Andersen, et al., 2012; Phillips, 2012). This is of particular note because most previous studies examining the genetic basis of pathogen response in nematodes have been conducted using *C. elegans,* with a common conclusion being that the amount of genetic diversity limits evolutionary diversification and the resultant functional bioactivity (Pujol, et al., 2008; Schulenburg & Boehnisch, 2008). Further, it has been shown that coevolution of a host organism with pathogenic bacteria drives self-fertilizing populations to extinction while selecting for increased outcrossing in mixed mating populations (Morran, et al., 2011). Presumably this arises out of a selection-imposed requirement for allelic and phenotypic variation. This suggests that despite the shared elements present in the *C. elegans* genome, the primary strategies employed for pathogen resistance may be unique. Given these evolutionary dynamics, the relevance of *C. elegans* as a general model for host-pathogen dynamics may be somewhat limited.

To overcome these limitations, I have created a model system using *Caenorhabditis remanei* and *Pseudomonas aeruginosa*. The nematode *C. remanei* is a soil-dwelling bacteriovore akin to *C. elegans*. It also is easily maintained and manipulated in lab environments, is transparent so that phenotypes can be easily scored, survives indefinitely when frozen, and has a short generation time that readily facilitates multi-generational examination of evolutionary genetic responses to controlled environments. Additionally, it possesses relatively facile genetic architecture and exhibits immune characteristics without any known orthologs of specific ligand-binding pathogen

receptors (pattern-recognition receptors, PRRs). In contrast to *C. elegans,* however, *C. remanei* is a gonochoristic (male-female) species. As such, the mode and tempo for its population demography is distinct from that of hermaphroditic nematode species, and the resulting amount of genetic diversity present in natural populations is extraordinarily high relative to *C. elegans* (Phillips, 2006).

Pseudomonas aeruginosa is a highly virulent and opportunistic mammalian pathogen that is also damaging to plants and invertebrates such as *C. remanei*. It is a frequent cause of death for individuals with cystic fibrosis, severe burns, and other forms of compromised immunity and is resistant to conventional treatments (Tan, et al., 1999). Under normal circumstances, the innate arm of the human immune system is capable of recognizing the presence of *P. aeruginosa* and initiating an effective immune response before an infection can become established. *Pseudomonas aeruginosa* is ubiquitous in soil but possesses a highly variable metabolism enabling it to survive in a wide variety of environments and to produce a number of environment-specific virulence factors (Cezairliyan, et al., 2013; Mahajan-Miklos, et al., 1999; Tan, et al., 1999). Presumably, the shared ecology between *P. aeruginosa* and *C. remanei* has led to historical host-microbe encounters between these organisms resulting in the presence of natural genetic variation at a relevant scale.

My model system is based on this interaction and takes advantage of the greater diversity present in *C. remanei* along with the variable metabolism of *P. aeruginosa* to investigate the genetic basis of pathogen resistance using an experimental evolution approach. In the lab environment, *P. aeruginosa* can kill *C. elegans* in either a slow infectious process (slow-kill) or by a toxin-mediated mechanism (fast-kill) depending on

the strain and culture conditions (Tan, et al., 1999; Mahajan-Miklos, et al., 1999). Slow-kill conditions require *P. aeruginosa* to be grown on nematode growth medium (NGM) and have a slower rate of death where fast-kill requires a high osmolarity medium and has faster kinetics. Observations indicate that on slow-kill media bacteria begin accumulating in the intestine of *C. elegans* approximately 36 hours after feeding begins (Tan, et al., 1999). Worms removed from slow-kill *P. aeruginosa* within 12 hours of exposure or placed on heat-killed bacteria with slow-kill media do not appear to exhibit any reduction in fitness (Tan, et al., 1999). Under fast-kill conditions, *P. aeruginosa* produces diffusible toxins and killing does not require contact with live bacteria (Mahajan-Miklos, et al., 1999). Recently, fast-killing has been shown to be mediated by production of phenazine-1-carboxylic-acid in a pH dependent manner (Cezairliyan, et al., 2013). In order to assess the evolutionary response of a natural population of *C. remanei* to *P. aeruginosa*, I performed a 40-generation study under slow- and fast-killing conditions, as well as their combination (Figure 1). The results from this work show that at least some of the components of these response pathways are independent from one another and that experimental evolution with *C. remanei* can be a powerful tool for exploring the genetic basis of pathogen resistance within natural populations.

CHAPTER II

RESULTS

***P. aeruginosa* is pathogenic to *C. remanei*.** When exposed to *P. aeruginosa* under slow-kill conditions, *C. remanei* recently collected from nature showed no obvious symptoms or increased mortality after 24 hours of exposure. Continued exposure results in a gradual cessation of pharyngeal pumping and loss of mobility followed by a significant increase in mortality by 72 hours (Figure 2). Differences between the pathogenic and control bacteria are most pronounced after 120 hours (5 days), with only half of the population exposed to *P. aeruginosa* remaining at that time (Figure 3; $X_1^2 = 67.5$, $p < 0.0001$). For simplicity, further tests of pathogen-induced mortality focus on this 5-day time point.

C. remanei populations are unable to persist for any significant length of time when continuously exposed to *P. aeruginosa* that have been induced to produce the fast-kill response (Figure 2). Subsequent fast-kill responses therefore involve an initial four hour exposure to the fast-kill environment, followed by an assessment of individual viability 24 hours later. Approximately 70% of the ancestral population died within 24 hours of exposure to *P. aeruginosa* under the fast-kill conditions (Figure 3, $X_1^2 = 135.0$, $p < 0.0001$).

***C. remanei* resistance to *P. aeruginosa* is an evolvable trait.** After 40 generations of experimental evolution in either slow- or fast-killing environments (denoted as SK or FK, respectively), the resulting *C. remanei* populations showed higher resistance to *P. aeruginosa* than the ancestral population when compared to the

appropriate matched control environment (Figure 4, SK: X_1^2 = 13.5, p = 0.0002; Figure 5, FK, X_1^2 = 373.1, p < 0.0001). There were no significant differences among replicates (SK, X_1^2 = 1.782, p = 0.1820; FK, X_1^2 = 1.195, p = 0.2743), so data was pooled across replicates in all subsequent analyses (see Tables 1 and 2 for replicate specific information).

Evolved resistance to one environment does not correlate to resistance in another. Individuals evolved in the fast-kill environment (FK) do not display any increase in survival when placed on slow-killing media (Figure 4, X_1^2 = 0.9, p = 0.3317). Control lines (Ctrl) survive significantly worse than either of the evolved resistance conditions (Figure 4, X_2^2= 42.8, p < 0.0001), and indeed perform marginally worse than the ancestors (Figure 4; X_1^2 = 3.4, p = 0.0665).

Survival under fast-kill environmental conditions increased significantly for both the slow-kill and control populations (Figure 5; X_2^2 = 200.9, p < 0.0001) but is still substantially less than that observed for the fast-kill populations (Figure 5; X_2^2 = 16.2, p = 0.0003).

The increased resistance to the fast-kill environment is not the result of adaptation to the high-osmolarity of the fast-kill inducing medium, as controls evolved under the same conditions but with the standard *E. coli* strain OP50 replacing *P. aeruginosa* (FKC) do not significantly differ in resistance from controls evolved under standard lab conditions (Figure 5, X_1^2 = 0.2, p = 0.6854). Although, slow-kill evolved populations (SK) survive fast-killing significantly better than ancestral individuals, they do not differ from lab adapted controls (Figure 5, X_2^2 = 4.2, p = 0.1212). This indicates that a portion

of the mechanism underlying resistance to fast-killing arises from exposure to standard lab conditions and is independent of interactions with *P. aeruginosa*. Moreover, it appears to have a negative influence on the mechanism giving rise to slow-killing resistance.

Evolution under alternating slow- and fast-killing environments. Lines evolved under an alternating exposure to slow- and fast-killing (SKFK) display no significant change in resistance in the slow-kill environment (Figure 4, $X_1^2 = 0.4$, $p = 0.5464$). In the fast-kill environment, SKFK populations have a significant increase in resistance compared to the ancestor (Figure 4, $X_1^2 = 52.7$, $p < 0.0001$), but the degree of resistance is significantly lower than all other evolved populations (Figure 7, $X_1^2 = 33.0$, $p < 0.0001$).

CHAPTER III

DISCUSSION

Contrasting kinetics of pathogenicity in the ancestral *C. remanei* population.

In slow- and fast-killing assays conducted with *C. elegans*, consumption of live bacteria was required for slow-killing while fast-killing occurred on PGS media on which *P. aeruginosa* had been grown and removed, suggesting that fast-killing is mediated by a diffusible toxin (Tan, et al., 1999; Cezairliyan, et al., 2013). The kinetics of pathogenicity in these studies is highly similar to the kinetics shown here with *C. remanei* (Figure 2; Tan, et al., 1999). Studies of *P. aeruginosa* mutants with attenuated killing abilities in *C. elegans* showed that many of the bacterial genes underlying pathogenicity in slow- or fast-killing are environment specific, primarily producing phenazines with relatively narrow and largely non-overlapping parameters for toxicity (Cezairliyan, et al., 2013; Mahajan-Miklos, et al., 1999; Tan, et al., 1999). From the bacterial perspective then, pathogenicity in the slow-killing and fast-killing environments clearly arises from distinctly different genetic and biochemical mechanisms. This is suggestive of a possibility for distinct genetic mechanisms to form the basis of response to slow-killing independently from fast-killing and vice versa in *C. elegans*. However, another possibility is that the basis of resistance in *C. elegans* is not specific to the environment or to toxins produced by *P. aeruginosa*, but is instead the manifestation of a generalized stress response shared across all environments. If this were the case, it is possible that the fast-killing environment is simply more taxing to the worm's system, thus generating the faster kinetic, or that slow-killing is equally taxing but requires more time to develop out of a bacterial dependence on quorum sensing or some other density-dependent

mechanism to initiate pathogenicity. Killing of *C. remanei* by *P. aeruginosa* also appears to take place through two distinct mechanisms. In the slow-killing environment exposure of *C. remanei* to *P. aeruginosa* for a minimum of 24 hours is required with significant mortality beginning at 72 hours of continuous exposure, whereas pathogenicity in the fast-kill environment requires only four hours of exposure to achieve 70% mortality at the 24-hour time point. Experimental evolution in *C. remanei* is therefore consistent with the suggestion that the genetic pathways underlying resistance to *P. aeruginosa* may operate independently from one another.

Evolutionary independence of resistance pathways. If the genetic mechanism underlying resistance consisted of a single stress response activated in both environments, the expected outcome would be for a strongly correlated and reciprocal resistant phenotype in the populations evolved under *P. aeruginosa* conditions. As my results show, evolution in the fast-kill environment did not confer any significant increase in resistance to the slow-kill environment. On the other hand, populations evolved under slow-kill conditions do show a significant increase in resistance to fast-killing when compared to the ancestral population. However, the degree of increase is significantly less than the fast-kill evolved group (Figure 5).

At first glance this appears to suggest that evolution in slow-killing conditions does offer some protection against fast-killing. However, both control populations, evolved in the same manner but with the OP50 strain of *E. coli* replacing *P. aeruginosa*, display the same level of resistance to fast-killing as the slow-kill evolved populations. This suggests that the increase in resistance to fast-killing displayed by the slow-kill

population is the result of adaptation to an effect that is not dependent specifically on *P. aeruginosa*. It seems plausible then that, because all of the aforementioned evolved lines (but not the ancestor) were routinely exposed to OP50 during the experimental evolution, this portion of increased fast-kill resistance is likely due to adaptation to OP50. This is supported by evidence demonstrating that the OP50 strain of *E. coli* is mildly pathogenic to *C. elegans* (So, et al., 2011). Like *P. aeruginosa*, OP50 is a gram-negative bacterium and thereby they have many genetic features in common, some of which have been shown to attenuate fast-killing virulence in *P. aeruginosa* mutants (Mahajan-Miklos, et al., 1999). For example, a bacterial gene, *MdoH*, identified to be important for full pathogenicity in fast-killing by *P. aeruginosa* is also present in OP50 (Mahajan-Miklos, et al., 1999). On the other hand, the small molecule phenazine-1-carboxylic-acid (PCA), a toxin necessary for fast-killing by *P. aeruginosa* (Cezairliyan, et al., 2013), is not produced by OP50. This suggests that fast-killing is a multifactorial process and that some of the sub-lethal pathogenic properties are shared between OP50 and *P. aeruginosa.* Moreover, the genes for the presumed primary toxic compounds produced by *P. aeruginosa* in the slow-killing environment, phenazines (and in particular the phenazine pyocyanin), are generally not present in the genus *Escherichia* and therefore are not found in OP50 (Reszka, et al., 2012; Kuznetsova, et al., 2012; Nowroozi, et al., 2012), lending credence to the hypothesis that the mode of toxicity employed by *P. aeruginosa* in the slow-kill environment may be wholly separate from OP50. This would account for the lack of any correlated increase in resistance from other environments. Taken together these data suggest the genetic pathways underlying resistance to slow-killing and fast-

killing by *P. aeruginosa* are largely independent of each other with an overlap in resistance to fast-killing mediated by adaptation to OP50.

An additional perspective on the evolutionary independence of the genetic pathways for slow-killing and fast-killing resistance comes from examination of the population evolved under alternating slow-killing and fast-killing conditions (SKFK). The SKFK populations display no significant increase in resistance to slow-killing but, despite having no exposure to OP50 during the experimental evolution period, do have a significantly increased resistance to fast-killing relative to the ancestral population. However, the level of resistance is significantly lower than the degree displayed by the populations evolved under slow-kill and control conditions (Figure 5). This is suggestive of an evolutionary antagonistic interaction between the slow-kill and fast-kill resistance pathways. Additionally, evidence from assays in *C. elegans* demonstrates that the transcription of antibacterial immune effectors is selectively repressed during infection with the fungal pathogen *Candida albicans* and that production of an antimicrobial peptide required for defense against fungal pathogens during infection by a bacterial pathogen increases susceptibility (Pukkila-Worley & Ausubel, 2012; Marsh, et al., 2011). This suggests that the worms are capable of distinguishing between pathogen types and coordinating responses among independent and antagonistic genetic pathways. Another possible factor is that the kinetics of pathogenicity in the slow-kill versus the fast-kill environment skewed the strength of selection in each environment to favor selection for fast-kill resistance. In other words, because fast-killing mortality was at 70% within 24 hours, non-resistant individuals exposed as L4s were likely to die before producing offspring whereas mortality in the slow-killing environment reaches 50% around day 5

giving individuals exposed as L4s ample time to produce offspring. It is also possible for both the kinetic skew and antagonistic interactions between pathways to be present.

As a whole this work suggests that *C. remanei* responds to virulence mechanisms of *P. aeruginosa* by at least two distinct genetic pathways. These two independent pathways are likely initiated in response to environment-specific factors through as-yet unknown interfaces. Also, these pathways appear to interact in an antagonistic fashion. In the context of host-pathogen interactions and bacteriovore feeding systems, this may reflect strategies evolved by the host to balance energy investment in antimicrobial defenses while addressing the capabilities of pathogenic bacteria to evade detection and predation. However, more work is needed to address this question.

CHAPTER IV

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, and Media. The *P. aeruginosa* strain PA14 (Rahme, et al., 1995) and the *E. coli* strain OP50 were used. Strains were grown at 37°C and the media used for bacterial culture and maintenance were Luria-Bertani broth (LB) (Miller, 1972) and King's broth (KB) (King, et al., 1954). PA14 was cultured on nematode growth medium lite (NGM lite) (US Biological catalog #N1005) for slow-killing assays or peptone-glucose-sorbitol (PGS) media (1% Bacto-Peptone, 1% NaCl, 1% glucose, 1.7% Bacto-Agar, 0.15 M sorbitol) for fast-killing assays (Mahajan-Miklos, et al., 1999; Tan, et al., 1999). OP50 cultured on NGM lite was used for a low-osmolarity control and OP50 cultured on PGS was used for a high-osmolarity control.

Nematode Strains. All *C. remanei* strains were maintained by standard culturing conditions on NGM lite agar with *E. coli* OP50 or *P. aeruginosa* PA14 as a food source at 20°C (Stiernagle, 1999). The strain used in this work was PX443, a derivative of 39 natural isolates collected from wood lice found in the same tract of forest detritus in the Koffler Scientific Reserve at Jokers Hill, King Township, Ontario by Rose Reynolds. The isolates were intercrossed in a scheme designed to equalize the genomic contribution from each strain to generate PX443.

Bacterial Virulence Survival Assays. Both the fast-killing and slow-killing assays were based on protocols described in Mahajan-Miklos et al. (1999). The fast killing assay was conducted by spreading 60 μL of liquid from a PA14 culture grown for

18 hours in KB on 11 cm plates containing peptone-glucose-sorbitol medium. After the culture was spread, plates were incubated at 37°C for 24 hours then placed at room temperature for 2-4 hours. Approximately 200 age-synchronized L4 individuals per plate were placed on fast kill media for survival or both PGS and NGM lite seeded with OP50 as controls and incubated at room temperature for 4 hours. The plates were then washed with S-basal buffer solution (Stiernagle, 1999), worms transferred onto NGM lite-OP50 plates, and surviving individuals counted 24 hours later. A total of three replicate plates per test condition were used.

The slow killing assay was conducted by spreading 20μL of liquid from a PA14 culture grown for 18 hours in KB on 3 cm plates containing NGM lite. The plates were then incubated at 37°C for 24 hours and placed at room temperature for 2-4 hours. A total of 90 age-synchronized L4 females per line were picked to said plates at a density of 3 females per plate. Resistance was scored as the fraction surviving after 5 days of exposure to the slow-kill environment. An individual was considered dead when a nose-touch failed to elicit any movement.

Experimental Evolution.

Selection in fast-kill environment. Two population replicates of *C. remanei* (PX443) were grown on NGM lite plates with OP50 as a food source for 40 generations. This population was exposed to PA14 on PGS solid media for 4 hours every other generation. High-osmolarity controls were passaged in the same manner but with OP50 substituted for PA14. A subset of each population was frozen every fifth generation for later analysis.

Selection in slow-kill environment. Two population replicates of *C. remanei* (PX443) were grown on NGM lite plates alternating PA14 and OP50 as the food source every other generation for 40 generations. Low-osmolarity (lab-adapted) controls were passaged in the same manner but with OP50 substituted for PA14. A subset of each population was frozen every fifth generation for later analysis.

Selection in slow-kill/fast-kill environments. Two population replicates of *C. remanei* (PX443) were grown on NGM lite plates with PA14 as the only food source for 40 generations. This population was also exposed to PA14 on PGS solid media for 4 hours every other generation. A subset of the population was frozen every fifth generation for later analysis.

Statistical Analysis. Slow-killing survival was calculated as the proportion of individuals surviving after five days of exposure. Individuals who had left the plates were removed from the data set. Fast-killing survival was calculated as the proportion of individuals surviving after 24 hours of removal from fast-killing relative to the number of individuals surviving after 24 hours of removal from control plates. Specific hypotheses were tested via categorical data analysis of weighted survival frequencies using JMP Pro10 (SAS Institute Inc, 2013). Differences among groups was tested using the chi-square resulting from the appropriate likelihood-ratio test.

APPENDIX

FIGURES AND TABLES

Figure 1: Experimental evolution design. Layout of environments followed by experimental evolution cycle.

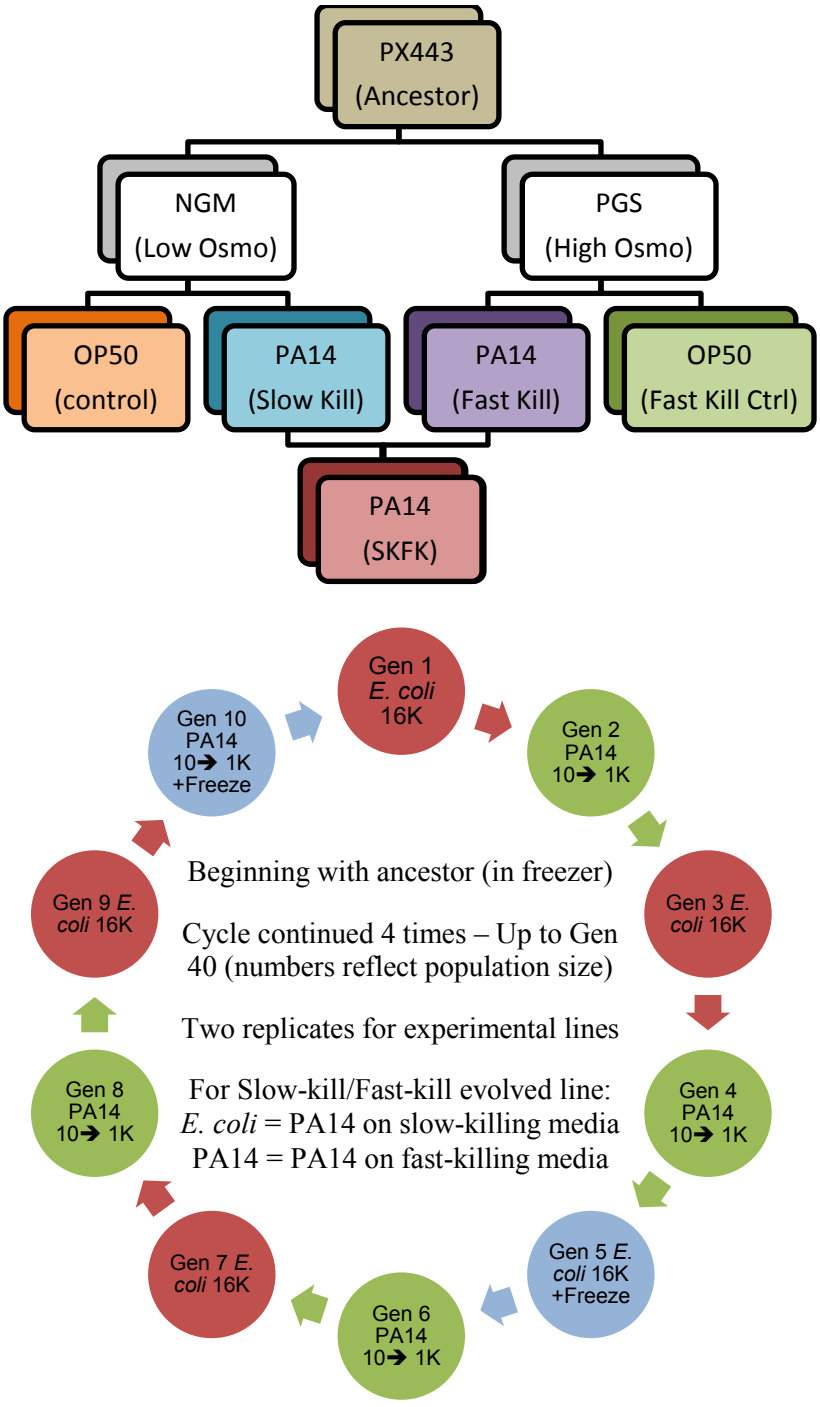

Figure 2: Survival kinetics of PX443 (Ancestor) L4 life-stage individuals in OP50 control, slow-killing, and fast-killing environments.

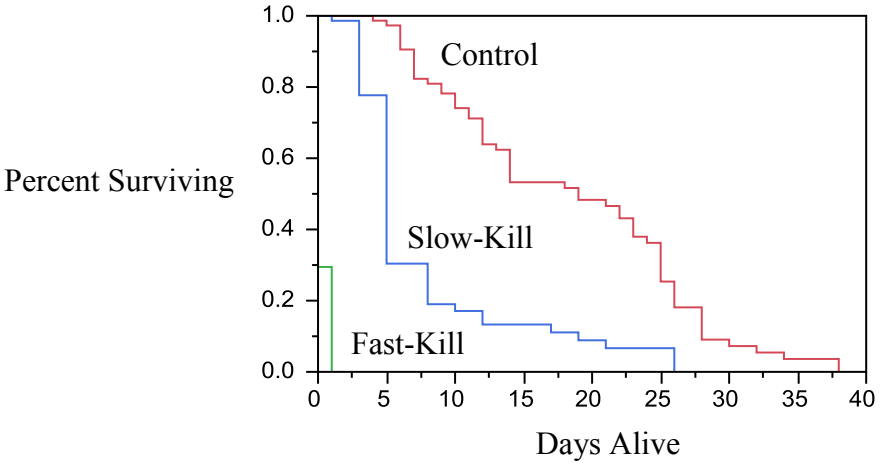

Figure 3: Percent survival of PX443 (Anc). Slow-killing measurements were taken at 5-days post-exposure and fast-killing measurement is 24 hours after completion of a 4-hour exposure. Error bars reflect standard error.

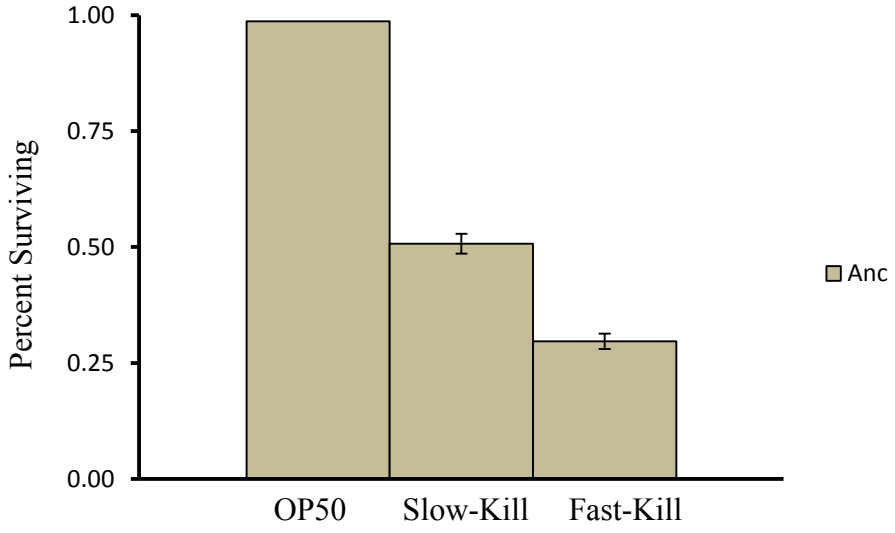

Figure 4: Slow-killing environment log fold change in survival of evolved populations relative to PX443. Error bars reflect standard error.

* = significant difference from PX443

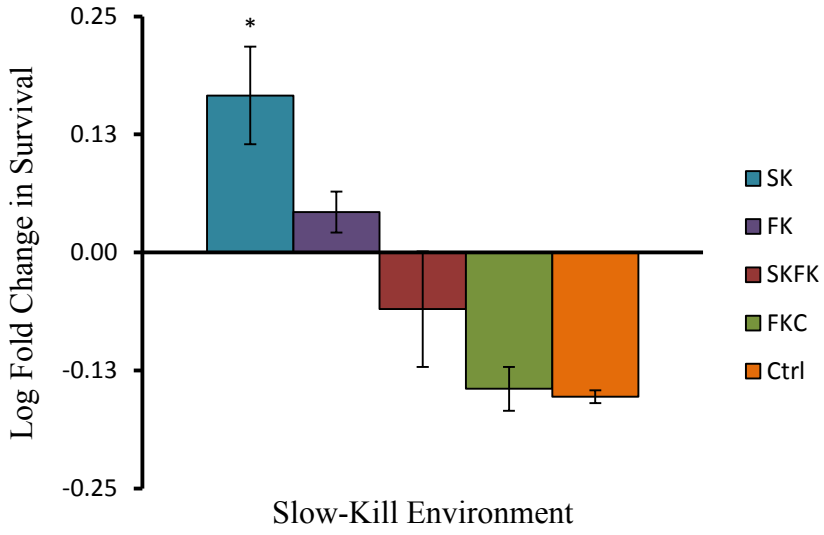

Figure 5: Fast-killing environment log fold change in survival of evolved populations relative to PX443. Error bars reflect standard error.

* = significant difference from PX443

† = significant difference from FK

‡ = significant difference from SKFK

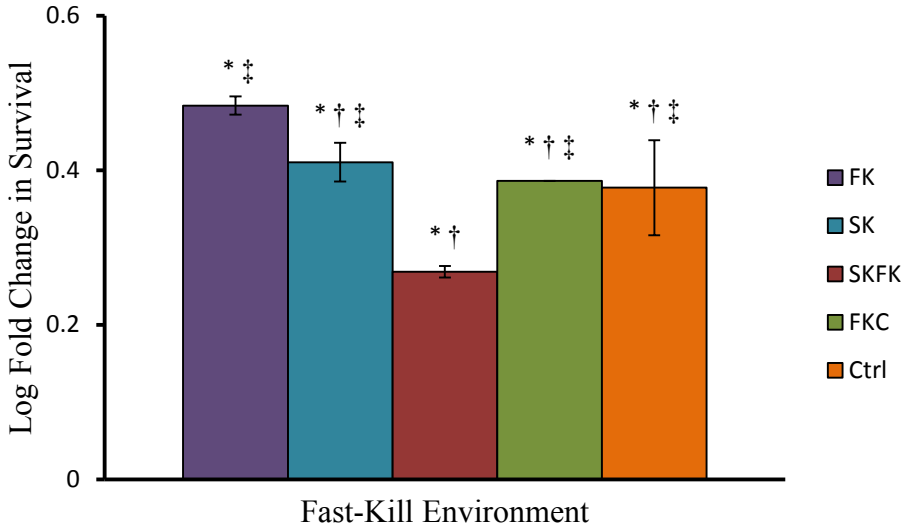

Table 1: Differences between replicates of experimentally evolved populations in fast-kill survival. Shaded cells have $p > 0.05$.

Fast-Kill ChiSquare Analysis	FK1	FK2	SK1	SK2	SKFK1	SKFK2	FKC1	FKC2	Ctrl 1	Ctrl 2	Anc
FK1											
FK2	$X^2_1 = 1.2$, $p = 0.2743$										
SK1	$X^2_1 = 37.3$, $p < 0.0001$	$X^2_1 = 52.5$, $p < 0.0001$									
SK2	$X^2_1 = 14.2$, $p = 0.0002$	$X^2_1 = 23.0$, $p < 0.0001$	$X^2_1 = 2.8$, $p = 0.0916$								
SKFK1	$X^2_1 = 99.6$, $p < 0.0001$	$X^2_1 = 119.1$, $p < 0.0001$	$X^2_1 = 30.7$, $p < 0.0001$	$X^2_1 = 38.7$, $p < 0.0001$							
SKFK2	$X^2_1 = 115.5$, $p < 0.0001$	$X^2_1 = 138.2$, $p < 0.0001$	$X^2_1 = 35.5$, $p < 0.0001$	$X^2_1 = 42.8$, $p < 0.0001$	$X^2_1 = 0.2$, $p = 0.6989$						
FKC1	$X^2_1 = 237.9$, $p < 0.0001$	$X^2_1 = 268.3$, $p < 0.0001$	$X^2_1 = 130.5$, $p < 0.0001$	$X^2_1 = 123.5$, $p < 0.0001$	$X^2_1 = 16.2$, $p < 0.0001$	$X^2_1 = 25.2$, $p < 0.0001$					
FKC2	$X^2_1 = 263.8$, $p < 0.0001$	$X^2_1 = 298.8$, $p < 0.0001$	$X^2_1 = 147.4$, $p < 0.0001$	$X^2_1 = 128.8$, $p < 0.0001$	$X^2_1 = 11.6$, $p = 0.0006$	$X^2_1 = 20.5$, $p < 0.0001$	$X^2_1 = 1.5$, $p = 0.2146$				
Ctrl 1	$X^2_1 = 43.9$, $p < 0.0001$	$X^2_1 = 58.5$, $p < 0.0001$	$X^2_1 = 2.1$, $p = 0.1508$	$X^2_1 = 7.4$, $p = 0.0066$	$X^2_1 = 14.0$, $p = 0.0002$	$X^2_1 = 14.4$, $p = 0.0002$	$X^2_1 = 72.3$, $p < 0.0001$	$X^2_1 = 71.4$, $p < 0.0001$			
Ctrl 2	$X^2_1 = 27.3$, $p < 0.0001$	$X^2_1 = 38.3$, $p < 0.0001$	$X^2_1 = 0.1$, $p = 0.8018$	$X^2_1 = 2.6$, $p = 0.1082$	$X^2_1 = 19.3$, $p < 0.0001$	$X^2_1 = 19.9$, $p < 0.0001$	$X^2_1 = 77.4$, $p < 0.0001$	$X^2_1 = 75.7$, $p < 0.0001$	$X^2_1 = 0.9$, $p = 0.3470$		
Anc	$X^2_1 = 265.4$, $p < 0.0001$	$X^2_1 = 295.1$, $p < 0.0001$	$X^2_1 = 159.2$, $p < 0.0001$	$X^2_1 = 152.0$, $p < 0.0001$	$X^2_1 = 33.3$, $p < 0.0001$	$X^2_1 = 46.6$, $p < 0.0001$	$X^2_1 = 5.3$, $p = 0.0218$	$X^2_1 = 13.3$, $p = 0.0003$	$X^2_1 = 136.4$, $p < 0.0001$	$X^2_1 = 103.4$, $p < 0.0001$	

Table 2: Differences between replicates of experimentally evolved populations in slow-kill survival. Shaded cells have $p > 0.05$.

Slow-Kill ChiSquare Analysis	FK1	FK2	SK1	SK2	SKFK1	SKFK2	FKC1	FKC2	Ctrl 1	Ctrl 2	Anc
FK1											
FK2	X^2_1 = 0.3, p = 0.6350										
SK1	X^2_1 = 16.5, p < 0.0001	X^2_1 = 13.1, p = 0.0003									
SK2	X^2_1 = 12.0, p = 0.0005	X^2_1 = 9.2, p = 0.0024	X^2_1 = 0.2, p = 0.6461								
SKFK1	X^2_1 = 1.4, p = 0.2310	X^2_1 = 0.5, p = 0.4618	X^2_1 = 8.0, p = 0.0046	X^2_1 = 5.2, p = 0.0222							
SKFK2	X^2_1 = 0.1, p = 0.7940	X^2_1 = 0.5, p = 0.4629	X^2_1 = 18.4, p < 0.0001	X^2_1 = 13.7, p = 0.0002	X^2_1 = 2.1, p = 0.1475						
FKC1	X^2_1 = 5.5, p = 0.0189	X^2_1 = 8.0, p = 0.0047	X^2_1 = 39.8, p < 0.0001	X^2_1 = 32.0, p < 0.0001	X^2_1 = 12.2, p = 0.0005	X^2_1 = 4.3, p = 0.0380					
FKC2	X^2_1 = 4.9, p = 0.0272	X^2_1 = 7.2, p = 0.0073	X^2_1 = 37.9, p < 0.0001	X^2_1 = 30.4, p < 0.0001	X^2_1 = 11.2, p = 0.0008	X^2_1 = 3.8, p = 0.0524	X^2_1 = 0.02, p = 0.9030				
Ctrl 1	X^2_1 = 2.8, p = 0.0947	X^2_1 = 4.6, p = 0.0314	X^2_1 = 32.3, p < 0.0001	X^2_1 = 25.5, p < 0.0001	X^2_1 = 8.1, p = 0.0045	X^2_1 = 2.0, p = 0.1614	X^2_1 = 0.5, p = 0.4876	X^2_1 = 0.3, p = 0.5711			
Ctrl 2	X^2_1 = 0.8, p = 0.3849	X^2_1 = 1.8, p = 0.1775	X^2_1 = 24.2, p < 0.0001	X^2_1 = 18.5, p < 0.0001	X^2_1 = 4.2, p = 0.0399	X^2_1 = 0.4, p = 0.5470	X^2_1 = 2.2, p = 0.1355	X^2_1 = 1.8, p = 0.1740	X^2_1 = 0.6, p = 0.4203		
Anc	X^2_1 = 1.6, p = 0.2073	X^2_1 = 0.6, p = 0.4215	X^2_1 = 7.5, p = 0.0063	X^2_1 = 4.8, p = 0.0281	X^2_1 = 0.01, p = 0.9409	X^2_1 = 2.3, p = 0.1314	X^2_1 = 12.5, p = 0.0004	X^2_1 = 11.5, p = 0.0007	X^2_1 = 8.3, p = 0.0039	X^2_1 = 4.4, p = 0.0349	

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