EXPLORING RESISTANCE TO *PSEUDOMONAS AERUGINOSA* INFECTION

EXPLORING RESISTANCE TO *PSEUDOMONAS AERUGINOSA* INFECTION IN *CAENORHABDITIS ELEGANS*

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TITLE: Exploring resistance to *Pseudomonas aeruginosa* infection in *Caenorhabditis elegans*

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LAY ABSTRACT

The human microbiome is the collection of beneficial bacteria that live on and in the human body and are involved in maintaining health in a number of ways, such as protecting from infection by pathogenic bacteria. In this work, we investigate the role the human microbiome has on altering the severity of an infection. Here, we use the model organism *Caenorhabditis elegans*, a microscopic roundworm that can be infected and killed by many of the same bacterial pathogens that infect humans. This worm model is particularly beneficial to use for this project because they grow and die more rapidly than other animals used to study infection, facilitating the ability to identify protective species within a large collection of bacterial isolates. This study shows that microbially-produced vitamins play an important role in enhancing survival during an infection. By studying infection resistance in model organisms such as *C. elegans*, we can identify factors that may also be important in regulating resistance to infection in humans.

ABSTRACT

Commensal microorganisms that colonize host surfaces can modulate susceptibility to infection. This can occur through direct inhibition of pathogen growth via competitive exclusion, or by modulation of the host environment to prevent pathogen dissemination and infection-mediated damage. However, understanding the mechanisms underlying these interactions is challenging due to the complexity of the human microbiome. The model organism *Caenorhabditis elegans* allows for the study of individual host-microbiota interactions, given they are bacterivorous and can be maintained monoxenically on a bacterial strain of interest. Here, we investigate the influence of human respiratory tract microbiota isolates on *C. elegans* susceptibility to *Pseudomonas aeruginosa* infection. Identifying isolates that differentially regulate resistance to infection will provide insight into how particular strains isolated from the human microbiome may act synergistically with *P. aeruginosa* or improve infection outcomes.

A screen of isolates derived from the human respiratory tract was carried out using a liquid-based *P. aeruginosa* infection assay. Animals were first exposed to individual microbiotal isolates during development, prior to infection with a pathogenic strain of *P. aeruginosa*, PA14. This screen identified two non-pathogenic isolates of *P. aeruginosa* that increased survival during PA14 liquid killing, compared to animals pre-exposed to *E.*

coli OP50. This protective phenotype was also induced by other mildly pathogenic strains of *P. aeruginosa*, including a laboratory strain with genetically attenuated virulence, PAO1 Δ*vfr*, as well as the pathogen *Salmonella enterica.* This work details the bacterial factors and host pathways that may regulate *S. enterica* and *P. aeruginosa*-mediated protection from PA14 liquid killing.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
DAMP	damage associated molecular pattern
DIC	differential inference contrast
FITC	fluorescein isothiocyanate
FSHR	follicle stimulating hormone receptor
FOXO	forkhead box protein class O
GFP	green fluorescent protein
hpi	hours post infection
IGF	insulin-like growth factor
L1	larval stage 1
L2	larval stage 2
L3	larval stage 3
L4	larval stage 4
LB	lysogeny broth
MAPK	mitogen activated protein kinase
NGM	nematode growth medium
PAMP	pathogen associated molecular pattern
PI3K	phosphoinositide 3-kinase
PGS	peptone glucose sorbitol
PRR	pattern recognition receptor
RNAi	RNA interference
SARM	sterile alpha and TIR motif containing

SKM	slow killing media
SKL	slow killing liquid
TBDT	tonB-dependent transporter
TIR	toll/IL-1 resistance
TLR	toll-like receptor
tRNA	transfer RNA
TRITC	tetramethylrhodamin
UPR	unfolded protein response

DECLARATION OF ACADEMIC ACHIEVEMENT

PAO1 ∆*vfr* and PA14 *pilE::mCherry* were gifted to us by the Burrows laboratory. The respiratory microbiota library was kindly provided by the Surette laboratory. Nathan Bullen aided with generation of the *cobN* mutant. Unless otherwise stated, all other work was completed by Mercedes Di Bernardo.

CHAPTER 1. INTRODUCTION

The diversity of microorganisms that colonize host surfaces, collectively termed the microbiome, provide vital functions for the animals ~~in which~~ they inhabit (Sommer and Bäckhed 2013). These include aiding in digestion by providing enzymes that facilitate the breakdown of complex carbohydrates, synthesizing essential micronutrients such as vitamins, regulating immune development, and protecting against infection by pathogens (Fan and Pedersen 2020; Sommer and Bäckhed 2013; Jingyan Zhang, Holdorf, and Walhout 2017). To date, most microbiota studies have focused on the taxonomic characterization of these communities (Zimmermann et al. 2019). Understanding how the microbiome functions on a systems-level is challenging due to its complexity and heterogeneity, particularly in humans (Zimmermann et al. 2019). The interactions between host genetics, environmental factors, and the microbiome make it difficult to characterize the function of individual species and their roles in health and disease (Diot, Garcia-Gonzalez, and Walhout 2018). Instead, the effect of a particular microbiotal species ~~has~~ on a disease phenotype of interest can be studied using model organisms (Diot, Garcia-Gonzalez, and Walhout 2018).

The roundworm *Caenorhabditis elegans* is a multicellular model organism used to study host-microbe interactions, due to its short lifespan and reproductive time, the ability to generate large populations of germ-free

animals, and, importantly, its bacterial diet. As a bacterivore, wild *C. elegans* will forage microorganisms in decomposing plant material, whereas in the laboratory, *C. elegans* is maintained monoxenically on a standard diet of non-pathogenic *Escherichia coli* (Revtovich, Lee, and Kirienko 2019). Although *C. elegans* is propagated on an individual strain of *E. coli*, many bacterial species can support its growth (MacNeil and Walhout 2013). Different bacterial diets provide unique combinations of macro- and micro-nutrients, vitamins, and other molecules that may act as signals to shape its physiology (MacNeil and Walhout 2013). Thus, the bacterial diet of *C. elegans* provides similar functionality to that the human microbiome, and certain species can colonize the intestine in a similar manner (Kumar et al. 2019). *C. elegans* is a useful model to study the influence of individual bacterial species on aspects of host physiology including development, longevity, metabolism, and immunity (Kumar et al. 2019). This thesis will focus on how *C. elegans'* bacterial diet modulates susceptibility to infection.

1.1 The *C. elegans* microbiome and pathogen resistance

The natural microbiome of *C. elegans* is only starting to be understood; the few studies that exist focus on its taxonomic characterization, and the effects that selected species have on its life history traits (Dirksen et al. 2016, 2020; Kumar et al. 2019; Samuel et al. 2016). Like the human gut, the intestine of *C. elegans* is colonized with a diversity of bacterial species

that associate with the intestinal epithelium (Kumar et al. 2019). Many of these isolates have been cultured in the laboratory; these include species predominantly within the phyla *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* (Dirksen et al. 2016, 2020; Samuel et al. 2016). The majority of these species (~80%) support *C. elegans* growth similar to *E. coli* OP50 in terms of developmental rate and population density, and do not induce stress or pathogen reporter genes (Samuel et al. 2016). The remaining 20% slow developmental rate and induce expression of unfolded protein response (UPR) and oxidative stress response genes (Samuel et al. 2016). Samuel *et al*. (2016) selected twelve species that span the core nine families comprising the *C. elegans* microbiome to generate a defined 'model microbiome', available publicly for future studies (Dirksen et al. 2020; Samuel et al. 2016). Using co-culture experiments with these strains, they showed that mixing beneficial isolates with detrimental ones could suppress the pathogenic effects of the detrimental strain, suggesting effects exerted on the host by the microbiome are a result of complex community interactions (Samuel et al. 2016).

One benefit of using *C. elegans* as a model for host-microbe interactions is the ability to study individual species or strains in isolation. Individual species isolated from the *C. elegans* microbiome can protect against infection by the opportunistic pathogen *Pseudomonas aeruginosa*

(Montalvo-Katz et al. 2013). Two commensal species, *Pseudomonas mendocina* and *Bacillus megaterium,* extend longevity during feeding on pathogenic *P. aeruginosa* using distinct mechanisms (Montalvo-Katz et al. 2013). *B. megaterium* does so indirectly by reducing *C. elegans* fecundity, as sterility is associated with infection resistance, whereas *P. mendocina* exerts its protective effect through modulation of conserved innate immune signalling pathways that coordinate the expression of antimicrobial effector molecules (Miyata et al. 2008; Montalvo-Katz et al. 2013). Commensal *Pseudomonas* isolates also protect against *Bacillus thuringiensis* infection, a natural *C. elegans* pathogen that produces pore-forming toxins that damage the intestinal epithelium (Kissoyan et al. 2019). Two of these *Pseudomonas* isolates directly inhibit *B. thuringiensis* growth via the diffusible antimicrobial molecule Massetolide E (Kissoyan et al. 2019). The other commensal *Pseudomonas* isolates improve intestinal barrier function without inhibiting *B. thuringiensis* proliferation, indicating that less damage is exerted on the intestinal epithelium during infection despite the presence of the pathogen (Kissoyan et al. 2019).

Human-derived probiotic bacteria can protect *C. elegans* from infection ~~in~~ (Rangan et al. 2016; M. Zhou et al. 2014; Mengzhou et al. 2014). The lactic acid bacterium *Enterococcus faecium* protects from *Salmonella enterica* infection by reducing pathogen load in the gut during the course of infection

(Rangan et al. 2016). *E. faecium* produces a secreted peptidoglycan hydrolase that generates peptidoglycan fragments to activate TOL-1 mediated immunity required for the defense response against *S. enterica* (Rangan et al. 2016). Furthermore, probiotic lactobacilli can protect from Enterotoxigenic *E. coli* infection either by stimulating expression of the antimicrobial peptide genes *clec-60* and *clec-85*, or by decreasing toxin gene expression in toxigenic *E. coli* to reduce virulence of the pathogen (Mengzhou et al. 2014). Given the genetic tractability of both *C. elegans* and the bacterial species it feeds on, it is a powerful model to study mechanisms underlying pathogen resistance (Irazoqui, Urbach, and Ausubel 2010). Bacterial diet-induced protection from infection can occur through multiple mechanisms, including stimulation of the innate immune response, direct inhibition of pathogen growth, or by modulation of virulence gene expression.

1.2 Antimicrobial immunity in *C. elegans*

Many pathogens that infect humans also cause lethal infection in *C. elegans* (Kumar et al. 2019). These include the Gram-positive pathogens *Staphylococcus aureus* and *Enterococcus faecalis,* as well as the Gram-negative pathogens *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Serratia marcescens*, all of which infect the nematode gut (Dierking, Yang, and Schulenburg 2016; Ermolaeva and Schumacher 2014; Kumar et al.

2019). As *C. elegans* lacks specialized immune cells, it must rely on behavioural responses and innate immunity to respond to a variety of bacterial pathogens (18). The evolutionary conserved NSY-1-SEK-1-PMK-1 mitogen activated protein kinase (MAPK) cascade is a central pathway of the *C. elegans* innate immune system and coordinates the response to a broad spectrum of bacterial pathogens (Kim et al. 2002; Shivers et al. 2009). The PMK-1 pathway is regulated by the mammalian *SARM1* homolog *tir-1*, a Toll/IL-1 resistance (TIR) adaptor protein (Liberati et al. 2004). Activation of the PMK-1 pathway by TIR-1 results in expression of effector genes such as lysozymes and antimicrobial peptides that combat infection (Troemel et al. 2006).

The insulin/insulin-like growth factor (IGF) pathway is a highly conserved signaling pathway involved in regulating *C. elegans* lifespan, stress resistance, and innate immunity (Garsin et al. 2003). Under normal conditions, the *C. elegans* IGF receptor DAF-2 activates AGE-1/PI3K, which phosphorylates the FOXO transcription factor DAF-16 and results in its cytoplasmic retention (Ogg et al. 1997). Under stress-inducing conditions such as nutrient limitation and pathogen infection, downregulation of insulin signaling leads to DAF-16 nuclear translocation, where it promotes the expression of genes involved in longevity and the stress response (Evans, Chen, and Tan 2008; Ogg et al. 1997). Therefore, activation of DAF-16

results increased lifespan and tolerance to both abiotic and biotic stressors such as infection (Evans, Chen, and Tan 2008; Ogg et al. 1997). Together, PMK-1 and insulin signaling function in parallel to regulate innate immunity in *C. elegans* (Evans, Chen, and Tan 2008).

1.3 *C. elegans* detects pathogen-mediated damage to coordinate the immune response to *P. aeruginosa*

To detect pathogenic bacteria and initiate an appropriate immune response, animals have evolved mechanisms to detect pathogen-associated molecular patterns (PAMPs), such as peptidoglycan, outer membrane lipids, and flagellar proteins (Ishii et al. 2008). In metazoans, the receptors that detect PAMPs are known as pattern recognition receptors (PRRs), and the best-characterized examples of these are Toll-like receptors (TLRs) (Ishii et al. 2008). *C. elegans* possesses two TLR homologs, the TIR proteins *tol-1* and *tir-1*, and though neither mediate recognition of PAMPs, ~~they~~ each plays key roles in innate immunity (Liberati et al. 2004). TIR-1 acts upstream of the PMK-1 MAPK pathway, and TOL-1 is required for the immune response to combat *S. enterica* infection, as well as the avoidance response to *S. marcescens* (Tenor and Aballay 2008). Instead of direct detection of PAMPs, *C. elegans* has evolved ways to survey for disruption of normal homeostatic processes mediated by pathogenic bacteria (Dunbar et al. 2012a; McEwan, Kirienko, and Ausubel 2012; Miller et al. 2015; Reddy

et al. 2016). *P. aeruginosa* exotoxin A blocks protein translation by ADP-ribosylating elongation factor 2, and rather than direct detection of the toxin, inhibition of protein translation is detected which results in an increase in ZIP-2 protein levels (McEwan, Kirienko, and Ausubel 2012; Michalska and Wolf 2015). ZIP-2 is a bZIP transcription factor that activates the expression of defense-related genes to promote resistance to *P. aeruginosa* infection (Estes et al. 2010). Some ZIP-2 regulated genes are also downstream of PMK-1 regulation, whereas others are induced independent of PMK-1 (Estes et al. 2010). Therefore, ZIP-2-mediated surveillance immunity acts in tandem, as well as in parallel with p38 MAPK signaling in *C. elegans* (Estes et al. 2010). Another component of innate immunity responsive to intestinal damage caused by multiple pathogens, including *P. aeruginosa*, is the G-protein coupled receptor FSHR-1 (Miller et al. 2015; Powell, Kim, and Ausubel 2009). FSHR-1 positively regulates the expression of antimicrobial effector genes and stress-associated genes in response to oxidative stress, heavy metals, and infectious bacteria (Miller et al. 2015). Though the mechanism underlying the induction of FSHR-1 is not as well understood as that of ZIP-2, it is likely activated upon cellular damage given its dual role in regulating genes that respond to both xenobiotics and pathogens (Miller et al. 2015).

Surveillance of mitochondrial damage is another mechanism by which *C. elegans* detects the perturbation of core cellular processes by pathogens or toxins (Pellegrino et al. 2014; Y et al. 2014). Many bacterial toxins target the mitochondria, including the *P. aeruginosa* virulence factors cyanide, pyocyanin, and pyoverdine (Gallagher and Manoil 2001; Kirienko, Ausubel, and Ruvkun 2015; O'Malley et al. 2003). Disruption of mitochondrial function induces expression of heat shock proteins, chaperones, detoxification enzymes, and innate immune effector genes including the *zip-2* regulated gene, *irg-1* (Pellegrino et al. 2014; Tjahjono and Kirienko 2017; Y et al. 2014). Infection response gene 1, or *irg-1*, is induced specifically by pathogenic *P. aeruginosa* infection independent of *pmk-1* (Estes et al. 2010). Although its function in the defense response to *P. aeruginosa* or mitochondrial damage is currently unknown, its regulation suggests that *C. elegans* interprets mitochondrial damage as a result of infection or xenobiotic attack (Lui et al. 2014). Therefore, *C. elegans* stress and innate immune responses are intrinsically linked.

1.4 Mechanisms of *P. aeruginosa* infection in *C. elegans*

P. aeruginosa is one of the best-studied pathogens of *C. elegans* and can kill the nematode using several distinct mechanisms. Two agar-based methods can be distinguished by the time it takes to cause lethality in *C. elegans*, known respectively as fast killing and slow killing (Tan, Mahajan-

Miklos, and Ausubel 1999). When cultured on high-osmolarity peptone-glucose-sorbitol (PGS) medium, *P. aeruginosa* kills *C. elegans* via intoxication by secreted phenazine toxins, which occurs over several hours (Cezairliyan et al. 2013; Mahajan-Miklos et al. 1999). Conversely, slow killing is an infection-like process that occurs over several days when *P. aeruginosa* is cultured on a minimal medium, referred to as Slow Killing Medium (SKM) (Tan, Mahajan-Miklos, and Ausubel 1999). The mechanism of slow killing is likely multifaceted, as a number of virulence factor regulators (*gacA*, *kinB*) and quorum sensing genes (*lasR*, *pqsE*) are required for this type of killing (Kirienko et al. 2013). Host colonization is a key pathogenic determinant of slow killing, and throughout the course of infection bacterial cells accumulate in the *C. elegans* intestine and damage the intestinal epithelium (Irazoqui et al. 2010; Tan, Mahajan-Miklos, and Ausubel 1999). Accumulation of bacterial cells in the intestinal lumen causes bloating and can result in aversion behaviour via neuroendocrine signalling, resulting in worms selectively avoiding pathogenic *P. aeruginosa* lawns (Singh and Aballay 2019).

Finally, a third mechanism of *P. aeruginosa*-mediated killing of *C. elegans* has recently been described, and occurs when animals are exposed to pathogenic *P. aeruginosa* grown in liquid medium similar in formulation to SKM (Anderson, Revtovich, and Kirienko 2018; Kirienko et al. 2013). This

method of killing seems to employ a distinct mechanism from agar-based methods, as PA14 mutants with reduced fast killing or slow killing do not display virulence impairments in liquid killing (Kirienko et al. 2013). For example, a PA14 strain lacking phenazine toxin biosynthetic enzymes (Δ*phz)*, critical for intoxication in fast killing, is not attenuated in liquid killing (Kirienko et al. 2013). Additionally, the PA14 quorum-sensing mutant Δ*lasR* has reduced virulence in slow killing, but not liquid killing (Kirienko et al. 2013). Whereas slow killing is reliant on quorum sensing and host colonization, a previous study indicates that liquid killing employs a virulence mechanism independent of phenazine production or intestinal colonization (Kirienko et al. 2013). Kirienko *et al*. described iron sequestration by the siderophore pyoverdine as a key virulence factor involved *P. aeruginosa* liquid killing (Kirienko et al. 2013). Although liquid killing was abrogated in Δ*pvd* mutants, extending the exposure to *P. aeruginosa* in liquid still resulted in significant death, indicating there are multiple mechanisms of virulence that have yet to be elucidated (Kirienko et al. 2013).

Liquid killing is also dependent on the phosphatase function of KinB, a sensor kinase involved in regulating biofilm formation, motility, and virulence factor production in *P. aeruginosa* (Chand, Clatworthy, and Hung 2012; Damron, Qiu, and Hongwei 2009). KinB phosphorylates the response

regulator AlgB to induce the production of virulence factors involved in chronic infections, such as alginate, and leads to biofilm formation. KinB's phosphatase activity maintains AlgB in a dephosphorylated state, allowing for production of acute virulence factors, including toxins and proteases (Chand, Clatworthy, and Hung 2012). KinB phosphatase activity is required for liquid killing, but its kinase activity is dispensable, indicating an acute mechanism of virulence.

C. elegans response to liquid killing appears distinct to that of slow killing (Kirienko et al. 2013; Kirienko, Ausubel, and Ruvkun 2015). Whereas *pmk-1* and *daf-2* mutants exhibit increased susceptibility to slow killing, these mutants are more resistant to liquid killing (Garsin et al. 2003; Kirienko, Ausubel, and Ruvkun 2015; Troemel et al. 2006). The host response to liquid killing also relies on the hypoxia-inducible response regulated by HIF-1, crucial for survival under hypoxic conditions (Kirienko et al. 2013; Romney et al. 2011). HIF-1 dependence for survival during liquid killing is in concordance with the hypoxic crisis induced by the siderophore pyoverdine as described by Kirienko *et al.* (2013). Autophagy promotes survival in both solid and liquid killing, however, Kirienko *et al*. (2015) demonstrated a specific role for mitophagy during liquid killing, as fragmentation of the mitochondrial network is more severe in the liquid assay compared to slow killing (Kirienko, Ausubel, and Ruvkun 2015; Zou

et al. 2014). Further, loss of the mitophagy regulators *pink-1* and *pdr-1* result in sensitization to liquid killing, as well as to intoxication by purified pyoverdine (Kirienko, Ausubel, and Ruvkun 2015). Interestingly, *zip-2* mutants are sensitive to both liquid killing and slow killing, suggesting that ZIP-2 may connect pathogen and stress-response pathways.

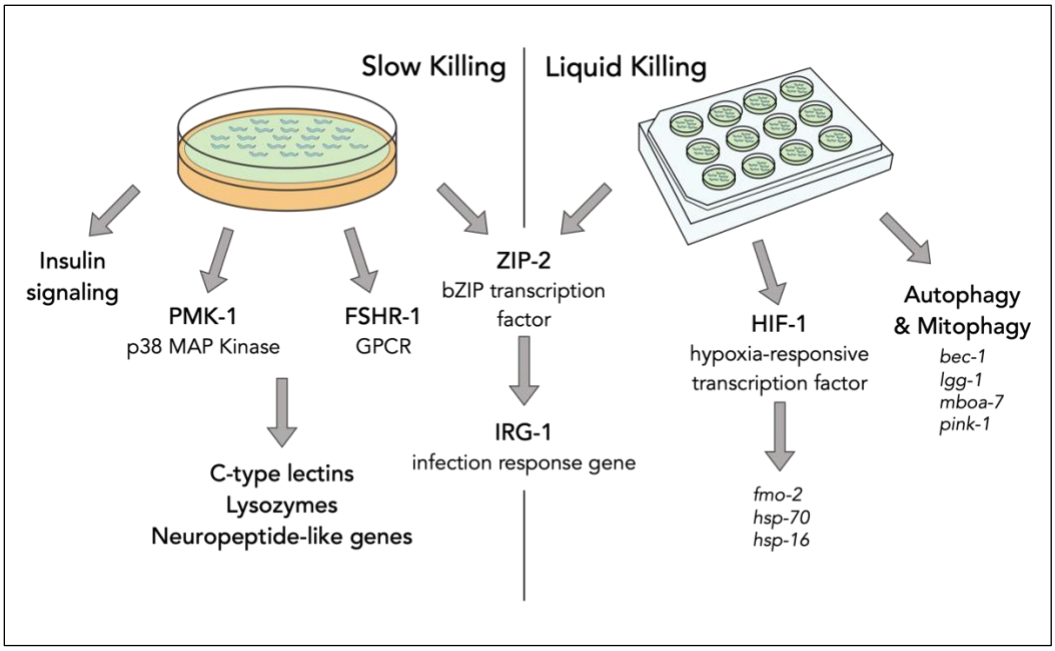

Figure 1. Summary of defense-response pathways initiated by *P. aeruginosa* in *C. elegans*. Solid and liquid killing initiate distinct innate immune and stress responses. Slow killing activates a conserved MAP kinase signaling pathway regulated by the p38 MAPK ortholog PMK-1, as well as the G-protein coupled receptor FHSR-1. These pathways act in parallel and confer broad-spectrum pathogen responses to both Gram-positive and Gram-negative pathogens via positive regulation of effector molecules such as lysozymes and antimicrobial peptides. Liquid killing activates pathways canonically associated with abiotic stressors such as hypoxia and heat shock. Exposure to *P. aeruginosa* in liquid also induces

mitochondrial damage, leading to expression of autophagic regulators, such as *bec-1* and *lgg-1*, and the mitophagic regulators *pink-1* and *pdr-1*. Both mechanisms of killing converge at the activation of ZIP-2, a bZIP transcription factor that controls the expression of a *P. aeruginosa*-specific infection response gene, *irg-1*, as well as other uncharacterized genes.

1.5 Project rationale

Given that *C. elegans* bacterial diet can modulate susceptibility to infection, we aimed to screen a respiratory microbiota library for isolates that can protect against *P. aeruginosa*-mediated killing, using the liquid killing assay to increase throughput. As *P. aeruginosa* is primarily a pulmonary pathogen in humans, screening for resistance-conferring bacteria from this library could potentially identify species from the lung microbiome able to reduce the virulence of *P. aeruginosa*, or microbiota-produced compounds that restrict its growth. Further, identification of isolates that modulate the *C. elegans* immune response could identify previously uncharacterized pathways involved in promoting resistance to infection.

CHAPTER 2. MATERIALS AND METHODS

2.1 *C. elegans* and bacterial strains

C. elegans and bacterial strains used in this study are listed in Table 1. *C. elegans* populations were maintained on *E. coli* OP50 lawns on Nematode Growth Medium (NGM; 0.25% peptone, 50 mM NaCl, 1.7% agar, 1 mM $CaCl_2$, 5 µg/ml cholesterol, 1 mM $MgSO_4$, 20 mM KH_2PO_4, 5 mM K_2HPO_4) plates at 20°C. Temperature-sensitive sterile *glp-4(bn2)* worms were propagated at 15°C. All bacterial strains listed in Table 1 were grown overnight in lysogeny broth (LB) at 37°C.

Table 1. *C. elegans* and bacterial strains used in this study.

Strain	Characteristics	Source
***C. elegans* strains**		
SS104	*glp-4 (bn2)*; temperature-inducible defect in germline proliferation when grown at the restrictive temperature of 25°C during larval development	Caenorhabditis Genetics Centre (CGC)
AU133	*agIs17* [*myo-2p::mCherry* + *irg-1p::GFP*]; expresses GFP in intestine and pharynx under control of the *irg-1* promoter when infected with pathogenic *P. aeruginosa*	Caenorhabditis Genetics Centre (CGC)
VL749	*wwIs24* [*acdh-1p::GFP* + *unc-119(+)*]; expresses GFP in the intestine and hypodermis under control of the *acdh-1* promoter	L.T. MacNeil

RB1434	*mmcm-1(ok1637)*. Homozygous. Null allele of *mmcm-1*	Caenorhabditis Genetics Centre (CGC)
Bacterial strains		
E. coli OP50		L.T. MacNeil
E. coli DH5α		L.T. MacNeil
E. coli SM10		J.C. Whitney
P. aeruginosa PA14		L.L. Burrows
P. aeruginosa PA14 + pBADGr-*pilE::mCherry*	Wild-type PA14 containing pBADGr-expressing *pilE* fused to mCherry.	L.L. Burrows
P. aeruginosa PAO1		L.L. Burrows
P. aeruginosa PAO1Δ*vfr*	Chromosomal deletion of *vfr*	L.L. Burrows
P. aeruginosa PAO1Δ*vfr*Δ*cobN*	Chromosomal deletion of *vfr* and *cobN*	This work
Comamonas DA1877		L.T. MacNeil
K. pneumoniae 32D9	Clinical isolate of *K. pneumoniae*	M.G. Surette
K. oxytoca 31D1	Clinical isolate of *K. oxytoca*	M.G. Surette
S. enterica sp. Typhimurium SL1344		B.K. Coombes
S. enterica sp. Typhimurium DM1	Clinical isolate of *S. enterica*	B.K. Coombes

2.2. *C. elegans* slow killing assay

C. elegans slow killing assays were performed as previously described (Tan, Mahajan-Miklos, and Ausubel 1999). *glp-4(bn2)* populations were maintained at 15°C on NGM plates seeded with *E. coli* OP50, and eggs were collected from gravid adults by hypochlorite treatment (Porta-De-La-Riva et al. 2012). Following egg collection, the population was synchronized

at the first larval stage then transferred to NGM plates seeded with *E. coli* OP50 or test bacteria for pre-exposure experiments. Animals were incubated at 25°C for approximately 40 h to obtain a population of sterile, early adult animals. During larval development, liquid cultures of *P. aeruginosa* were grown overnight in LB, and 200 µl was transferred to 6 cm Slow Killing Media plates (SKM; 0.35% peptone, 50 mM NaCl, 2% agar, 1 mM $CaCl_2$, 5 µg/ml cholesterol, 1 mM $MgSO_4$, 20 mM KH_2PO_4, 5 mM K_2HPO_4), ensuring the culture was spread to the edges of the plate. Lawns were grown at 37°C for 24h. Early adult *glp-4(bn2)* animals were washed three times in M9 buffer, then approximately 80-100 animals were transferred to *P. aeruginosa* lawns, as well as an *E. coli* OP50 control. Plates were incubated at 25°C and scored for live worms daily. Animals unresponsive to touch with a platinum wire were considered dead, then removed from the plate. Survival curves were generated using GraphPad Prism Software Version 9.2.0 and statistical significance between treatment conditions was obtained using the Log-rank (Mantel-Cox) test.

2.3 *C. elegans* liquid killing assay

C. elegans liquid killing assays were performed as previously described, with modification (Anderson, Revtovich, and Kirienko 2018). Early adult populations of *glp-4 (bn2)* animals were prepared as described in Chapter 2.2, replacing M9 buffer with S-basal buffer (5.85 g NaCl, 1 g K2HPO4, 6 g

KH_2PO_4 dissolved into 1 L of water and sterilized by autoclave, supplemented with 5 µg/ml cholesterol). Twenty-five microliters containing approximately 50 washed animals were transferred to wells of a 96-well plate, with four replicate wells for each pre-exposure condition. During larval development, liquid cultures of *P. aeruginosa* PA14 and *E. coli* OP50 were grown overnight in LB, then 350 µl ~~of~~ each were transferred to 10 cm SKM plates. Lawns were grown at 37°C for 24h, then cells were scraped from each plate into 2 ml of S-basal buffer and optical density (OD_{600}) was measured using a spectrophotometer. The cell suspensions were diluted in S-basal to 4X the optical density used in the assay. Fifty microliters of Slow Killing Liquid (SKM; 0.35% peptone, 50 mM NaCl, 1 mM $CaCl_2$, 5 µg/ml cholesterol, 1 mM $MgSO_4$, 20 mM KH_2PO_4, 5 mM K_2HPO_4) was added to each well, followed by 25 µl of the 4X cell suspensions for a final well volume of 100 µl. Plates were sealed with gas-permeable membranes and incubated at 25°C. At 48 and 72 h viability was scored by visual examination using a dissecting microscope. GraphPad Prism Software was used to plot and analyze survival data. One-Way ANOVA with Dunnett's multiple comparisons test was used to determine significant differences in survival between treatment conditions at each time point.

2.4 Preparation of cell-free supernatants

Cultures of each bacterial strain were grown in 3 ml LB overnight at 37°C with shaking. For supernatant collection, cultures were pelleted by centrifugation, then the supernatant was aspirated and sterilized via passage through a sterile syringe filter with a pore size of 0.22 µm. Sterile cell-free supernatants were transferred to fresh lawns of *E. coli* OP50 grown on 6 cm SKM, then allowed to dry before transfer of synchronized L1 animals for larval development.

2.5 Molecular cloning procedures

Primers used for cloning and genotyping are listed in **Table 2**. Genomic DNA was isolated from *P. aeruginosa* PAO1 using an InstaGene™ Matrix kit (Bio-Rad). To generate the *cobN* deletion allele, ~400 bp 5' and 3' of the gene was amplified, then spicing-overlap extension PCR (SOE-PCR) was used to assemble the two amplicons into a contiguous mutant allele. Primers used to amplify the flanking regions contained HindIII and XbaI restriction sites and restriction enzyme cloning was used to transfer the mutant allele to pEXG2.

For genotyping worm strains, individual animals were lysed by proteinase K digestion at 55˚C for 1.5hrs and heat inactivated at 95˚C for 15 minutes. Lysates were used to amplify the gene of interest to identify deletion alleles.

For *glp-4(bn2)* genotyping, the *glp-4* amplicons were digested with BmgB1 (NEB) to identify the *bn2* point mutation. Digested products were 413 bp and 494 bp, whereas the undigested, WT product was 907 bp.

Table 2. Primers and plasmids used in this study*

Primer name	**Sequence (5'→3')**	
Bacterial primers		
cobN HindIII	TGTTAAGCTA**AAGCTT**GAGCTGAACAAGGGCTGCCCATGAA	
cobN XbaI	TGTTAAGCTA**TCTAGA**TCCGTATCCGCACTGACCACCCT	
cobN overlap F	AACTCGAGCCGCAAGCATGCTGAAGAAGAGGAATAGCGGAGCGGCATG	
cobN overlap R	TTCAGCATGCTTGCGGCTCGAGTTCAGGTGCATGGACGTTCTACCGGT	
cobN external check F	ACCGAGCGGATTCTCCGCGAC	
cobN external check R	TGAAGATCGTCGAGGCGAGCCG	
cobN internal check	CGCTGCAGAGGATCACCAGCTC	
vfr check F	GCGCTGTCGAACCCCAT	
vfr check R	CGTCAGGCATATTCTTTCTTTC	
***C. elegans* primers**		
mmcm-1 F	TTGTACTCTGTGCGGGGTGTC	
mmcm-1 R	CGGAACGACACGCGCTTTATATG	
glp-4 F	CCACGGTAGAGACCCTTCTCCTTG	
glp-4 R	GACCTTGGTTGAGGCTAAGCGACTAC	
Plasmid	**Features**	**Source**
pEXG2	Gateway vector used for two-step allelic exchange; Gm^r, *mob*$^+$*, sacB, lacZ*	J.C. Whitney

pBADGr	Broad-host range vector used for expression of transgenes; Gm^r, *mob*$^+$, *PBAD-araC*	L.L. Burrows
pBADGr-*pilE::mCherry*	pBADGr expressing fluorescent *pilE* fusion construct	L.L. Burrows

*** Restriction enzyme cut sites are bolded**

2.6 Generation of the *cobN* mutant by allelic exchange

Two-step allelic exchange (Hmelo et al. 2015) was used to delete *cobN* in *P. aeruginosa*. The deletion allele construct in pEXG2 was transformed into competent *E. coli* XL1 by heat-shock transformation. Colony PCR was used to screen for presence of the insert, then plasmids were isolated and verified using Sanger Sequencing (GeneWiz, South Plainfield, NJ). Verified allelic exchange constructs were transformed into donor *E. coli* SM10 and conjugated with parent *P. aeruginosa* PAO1 Δ*vfr*. Matings were plated on LB supplemented with 30 µg/ml gentamicin and 25 µg/ml Irgasan and grown overnight at 37°C to select for *Pseudomonas* colonies that have integrated pEXG2 into the chromosome. To isolate a double-crossover event, colonies were picked into LB broth without antibiotic, grown at 37°C for ~1 h, then streaked onto no-salt LB plates supplemented with 5% sucrose to select against merodiploids. Plates were incubated at 30°C overnight, colonies were then patched onto LB agar, and LB agar supplemented 30 µg/ml gentamycin. Colony PCR was used to screen for the mutation. Colonies that

produced amplicons of the correct deletion size and did not grow on LB + gentamycin were stored at -80°C in 25% glycerol.

2.7 Fluorescence microscopy

Images were taken with a Nikon Eclipse epi-fluorescence microscope using differential interference contrast (DIC) and fluorescence microscopy. Samples were imaged through Plan Apo 10X (NA = 0.45) and 20X (NA = 0.75) lenses using FITC or TRITC filters. All image acquisition was carried out using Nikon NIS-Elements Advanced Research software (Version 4.51.00), and quantification of fluorescence intensity was carried out using ImageJ software (Version 1.53). For visualization of *irg-1p::GFP* expression, the liquid killing assay was set up as described in Chapter 2.3, then 20 h post infection animals were washed three times with M9 buffer, animals were transferred to 2% agarose pads for imaging. For visualization of *acdh-1::pGFP* expression, synchronized L1s were transferred to lawns of each test bacteria, then picked onto agarose pads 24 and 48 h later for visualization. Worms were anaesthetized with 5 µl 12 mM levamisole and a coverslip was placed on top of the agarose pad once the worms stopped moving.

2.8 Bacterial colonization assays

To visualize intestinal colonization of PA14, the liquid killing assay was set up as described in Chapter 2.3, using a strain of PA14 constitutively expressing mCherry (PA14 + pBADGr-*pilE::mCherry*). Twenty-four hours post infection, animals were washed three times with M9 buffer and transferred to 2% agarose pads for imaging. Worms were anaesthetized with 5 µl 12 mM Levamisole, then a coverslip was placed on top of the agarose pad once the worms stopped moving. Fluorescent images were taken as described in Chapter 2.8. Intestinal colonization was quantified using a previously described method, with modifications (Portal-Celhay and Blaser 2012). The liquid killing assay was set up as described in Chapter 2.3, using a strain of PA14 containing a plasmid harbouring gentamicin resistance to allow for selective growth during CFU quantification. Approximately 50 animals were transferred from the liquid killing assay 24 hours post infection into 1.5 ml Eppendorf tubes, then allowed to settle by gravity. The supernatant was removed, and animals were washed once with 1 ml M9 buffer + 100 µg/ml ampicillin to dilute the initial concentration of PA14 in the suspension. To remove surface-attached bacteria, animals were transferred to 6 cm SKM plates containing 100 µg/ml ampicillin and allowed to crawl for 30 minutes. Animals were washed from plates with 1 ml 12 mM levamisole in M9 buffer (LM buffer) to prevent pharyngeal pumping and expulsion of intestinal contents, then washed three times with 100 µl

LM buffer + 100 µg/ml ampicillin, then three times in M9 buffer alone. Approximately ten sterile silica carbide beads were transferred to each tube, then vortexed at maximum speed for 1-minute intervals until all animals were sufficiently lysed, as determined by visual inspection under a dissecting microscope. The lysis products were serially diluted in M9 buffer, plated on supplemented with 30 µg/ml gentamicin, and incubated at 37°C overnight. Prior to lysis, the supernatants from the final wash were also diluted and plated to ensure sterility of the solution. The following day, colonies were counted and colony forming units per sample were quantified.

2.9 *C. elegans* genetic crosses

Genetic crosses were performed according to standard practice (Brenner 1974). *mmcm-1(ok1637)* animals were outcrossed twice to N2 prior to crossing into a *glp-4(bn2)* background. Briefly, 20-30 *glp-4(bn2)* males and four *mmcm-1(ok1637)* hermaphrodites were picked onto 3.5 cm NGM plates seeded with OP50. The plates were maintained at 15°C to allow for reproduction of *glp-4(bn2)* animals. After approximately five days, a few young F1s were picked onto individual 3.5 cm plates. Once eggs were laid, F1s were lysed and genotyped by PCR to identify *mmcm-1(ok1637)*/+ heterozygotes. Forty F2s were picked from a selected heterozygote F1 onto individual plates, and once eggs were present on the plate, were lysed and genotyped by PCR. For all *mmcm-1(ok1637)* homozygotes the *glp-4*

genotype was determined. The *bn2* allele contains a point mutation that results in the generation of a BmbGI restriction site, so amplicons were digested to identify *mmcm-1(ok1637); glp-4(bn2)* double mutants. To verify the temperature-sensitive sterility, *mmcm-1(ok1637); glp-4(bn2)* animals were incubated at the restrictive temperature of 25°C until adulthood.

CHAPTER 3. RESULTS

3.1 Optimization of a *C. elegans*-PA14 Liquid Killing Assay

The slow killing assay was the first *P. aeruginosa* infection assay developed for *C. elegans*, and is the prototypical assay used to study *P. aeruginosa* virulence in this model (Park, Jung, and Lee 2017). However, this assay is performed on solid media and is not feasible for use in large-scale screening projects, as killing takes place over the span of multiple days and scoring survival is labour-intensive and time-consuming. Liquid-based infection assays can be carried out in multi-well plate formats, which allow for many conditions to be screened in one experiment. These assays have been used to identify bacterial factors required for pathogenesis, and to identify anti-infective compounds against *P. aeruginosa* (Anderson, Revtovich, and Kirienko 2018; Garvis et al. 2009; Kirienko et al. 2013). A previously published liquid killing assay was used to determine the optimal initial bacterial density that allows for sufficient pathogen-mediated killing within 48 h (Anderson, Revtovich, and Kirienko 2018; Kirienko et al. 2013). Killing was dose-dependent, as higher densities of PA14 resulted in more rapid killing **(Fig 2A)**. The majority of killing took place within 48 h, and only very high concentrations of *E. coli* OP50 were able to cause host death, consistent with previous findings **(Fig 2A)** (Kirienko et al. 2013). A low initial starting inoculum of PA14 (OD_{600}=0.03) was reportedly sufficient to cause significant host death 48 h post-infection compared to OP50, however, we

found that only ~10% of animals were killed with this initial density of PA14 **(Fig 2A)** (Kirienko et al. 2013). To cause > 50% host death within 48 h, the OD_{600} of PA14 had to be four times higher than previously reported **(Fig 2).** This could suggest our PA14 strain exhibits reduced virulence compared to the laboratory strain used in the referenced work. This is not unexpected, as different laboratory strains of *P. aeruginosa* PAO1 produce variable types and amounts of secreted products involved in virulence such as pyoverdine, pyocyanin, *Pseudomonas* quinolone signal (PQS), and exopolysaccharides (Chandler et al. 2019).

For all subsequent assays performed while screening the microbiota library for resistance-conferring isolates, the lowest initial density that killed the most animals within 48 h was used (OD_{600}=0.125). It was important that the density of PA14 was high enough to allow for sufficient killing, but not too high such that potential effects of a treatment would be masked by sheer density of pathogenic cells (Conery et al. 2014).

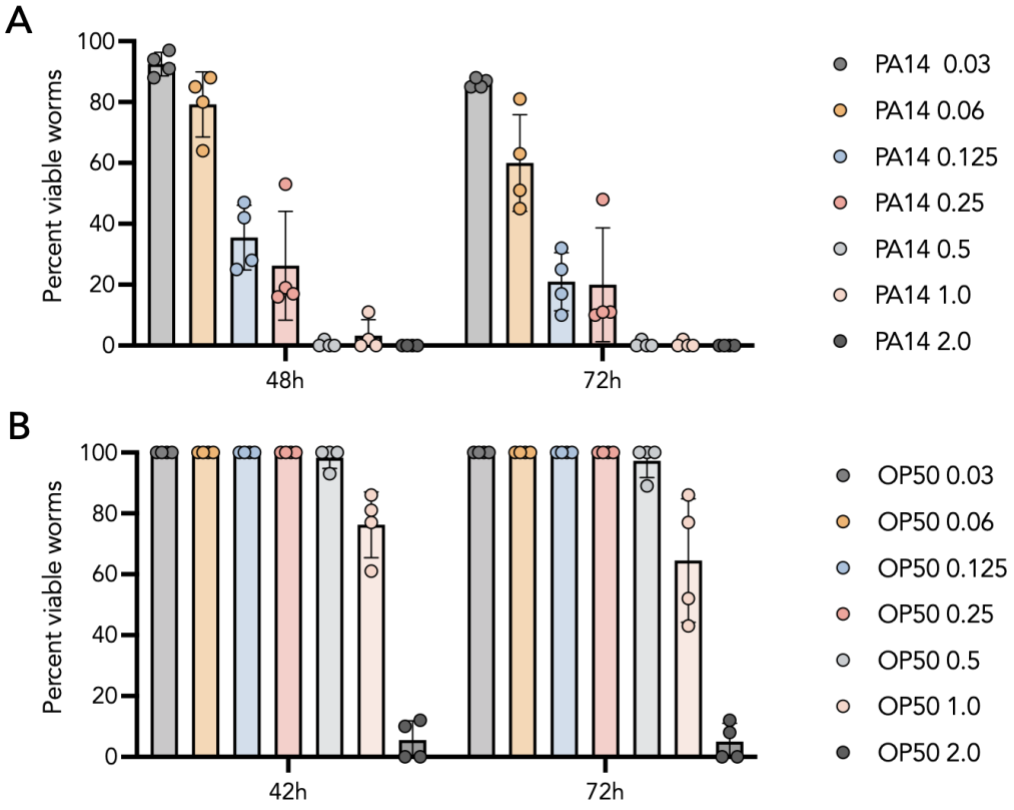

Figure 2: Killing of *C. elegans* by PA14 in liquid depends on initial density of bacterial cells. A. Host death increases with increasing initial OD_{600} of PA14. Low densities (OD_{600}=0.03-0.06) do not induce substantial host death, whereas very high densities result in nearly 100% population death after 48 h (OD_{600}=0.5-1.0). **B.** Only very high densities of OP50 are sufficient to cause host death (OD_{600}=1.0-2.0). Each point represents a single well containing approximately 50 animals.

Colonization of the *C. elegans* intestine is a major pathogenic determinant of slow killing, whereas liquid killing has been reported to occur independent of intestinal colonization (Kirienko et al. 2013). Because a much higher density of pathogenic cells was required to cause significant host death in

our assay compared to a previous study, intestinal colonization during liquid killing conditions was explored. Fluorescently-labelled PA14 (PA14 + pBADGr-*pilE::mCherry*) was used in the liquid killing assay, and animals were imaged 24 h post infection. Even at the lowest initial concentration tested, faint fluorescent signal was detected in the intestine, which increased with increasing bacterial density **(Fig 3A).** Individual fluorescent cells were visualized in the posterior end of the intestine, showing that intact cells are indeed present in the intestinal tract during liquid killing **(Fig 3E).** Given that fluorescent signal increased with density of PA14 cells, this suggested that our strain of PA14 colonizes the intestine in liquid killing conditions **(Fig 3)**. Further, this indicated there could be multiple mechanisms contributing to liquid killing, depending on the virulence traits of the strain of *P. aeruginosa* used in the assay.

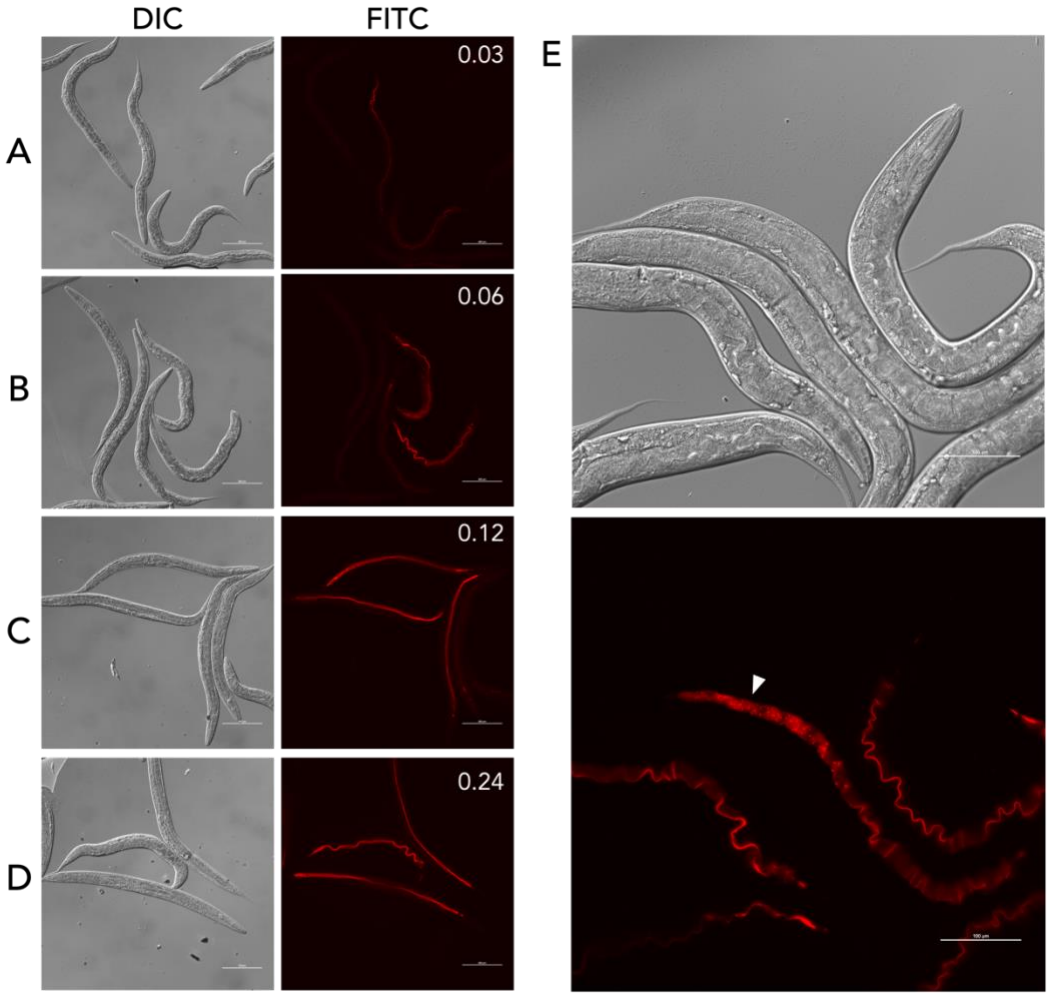

Figure 3: Intestinal colonization occurs even at low initial density of PA14 in the liquid killing assay. A – D. Fluorescent signal in the intestine increases with density of PA14 cells. Animals were aspirated from the liquid killing assay 24 hpi and imaged using DIC and the FITC filter. Scale = 200µm. **E.** Intact PA14 cells are present in the posterior end of the intestine. The white arrow denotes the region of the intestine where intact cells can be visualized. Scale = 100µm.

3.2 Bacterial diet alters *C. elegans* susceptibility to *P. aeruginosa* liquid killing

To identify microbiotal isolates that can protect *C. elegans* from *P. aeruginosa*-mediated killing, animals were grown on lawns of each isolate during larval development, then transferred to the liquid killing assay. This allowed the exposure window to occur prior to infection, which is typically carried out at the L4 stage (Anderson, Revtovich, and Kirienko 2018). However, different bacterial diets can alter developmental rate in *C. elegans* (MacNeil and Walhout 2013). Given the diversity of bacterial species present in the respiratory library and their lack of characterization in *C. elegans,* it was feasible to expect that some isolates might affect developmental rate. To mitigate this, L1s were transferred to mixed lawns of each isolate and OP50, as this method has previously been used to preserve developmental rate on pathogenic bacteria, such as *Microbacterium nematophilum* (Gravato-Nobre et al. 2005). Prior to setup of the liquid killing assay, the developmental stage of the animals was confirmed to ensure age was not a confounding factor in the survival outcome. Isolates that resulted in asynchronous populations, or populations where most animals were younger or older than L4, were not used for the infection assay. Using this method, 49 isolates from the respiratory library were screened, and the percent viability of worms pre-treated with each

microbiotal isolate was compared to worms pre-treated only with OP50 **(Fig 4A).**

Pre-exposure to most isolates reduced survival during PA14 infection, compared to animals fed only OP50 during larval development; 39 out of the 49 isolates decreased viability, whereas only three significantly increased viability **(Fig 4A).** Most genera could be categorized as either protective or detrimental, with only two, *Rothia* and *Dolosigranulum*, having isolates with opposing effects; that is, some isolates were protective, and others within the genus promoted killing **(Fig 4B)**. Following the screen, the three most protective isolates were re-tested in pure culture. Upon repetition, two isolates of *Pseudomonas aeruginosa*, denoted as ‘A9’ and ‘C10’, significantly increased survival, whereas the third protective isolate, *Pantoea sp.,* only did so marginally **(Fig 4C)**.

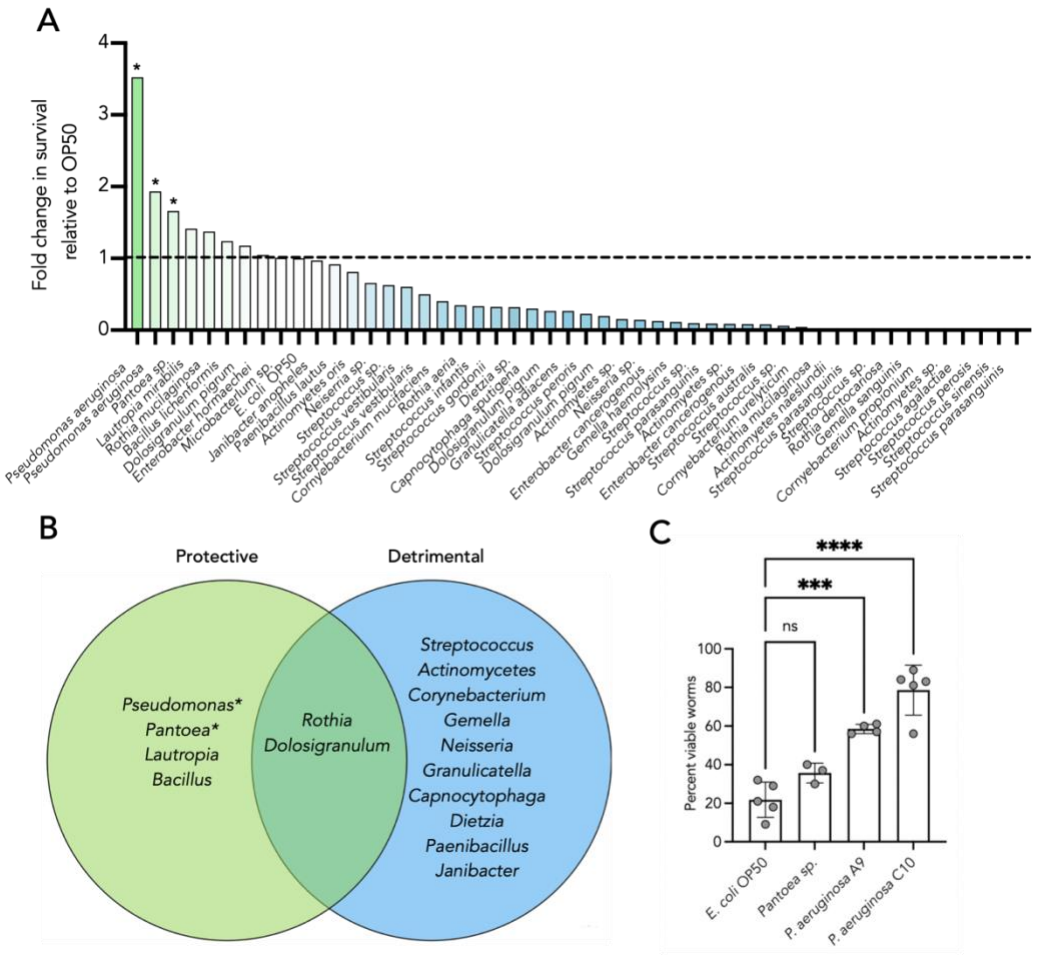

Figure 4: Pre-treatment with human respiratory microbiome isolates alter *C. elegans* survival in a liquid-based *P. aeruginosa* killing assay. **A.** Most isolates in the respiratory library negatively impacted survival outcomes in the liquid killing assay. Percent survival was normalized to worms only fed with OP50 during larval development, represented by a dashed line. Asterisks indicate isolates that significantly increased survival compared to OP50-pre-treated worms. **B**. Genera were grouped into 'protective' if survival was greater than 1, and 'detrimental' if it was less than 1. **C**. Two *P. aeruginosa* isolates from the respiratory library provided significant protection from liquid killing. Each data point represents a well containing approximately 50 animals. Asterisks indicate isolates that

significantly increased survival compared to OP50-pre-treated worms by One-Way ANOVA with Dunnett's multiple comparisons test. *** $p<0.001$, **** $p<0.0001$.

When fed pathogenic bacteria such as virulent strains of *P. aeruginosa, C. elegans* larvae will arrest development (Samuel et al. 2016). The developmental stage of animals was visually checked prior to transfer to the liquid killing assay and isolates that did not produce a synchronous population of L4s were discarded from the screen, therefore the two protective *P. aeruginosa* isolates did not alter developmental rate. Even when fed pure cultures, animals developed at a rate comparable to OP50-fed worms, indicating these isolates are not highly virulent to *C. elegans*. To determine if the protective effect is generalizable to mildly pathogenic strains of *P. aeruginosa,* two other laboratory strains were tested: PAO1, and a PAO1 mutant with reduced virulence due to deletion of the gene encoding a master regulator of virulence factor production, *vfr* **(Fig 5A)**. Pre-treatment with PAO1 did not increase survival in the liquid killing assay compared to OP50-fed animals, whereas pre-treatment with PAO1 Δ*vfr* did **(Fig 5A and C)**. This is likely due to wild-type PAO1 exerting infection-mediated damage during pre-treatment, whereas PAO1 Δ*vfr* did not do so to the same extent, allowing for exposure to a protective factor without compounding damage due to infection during the developmental period.

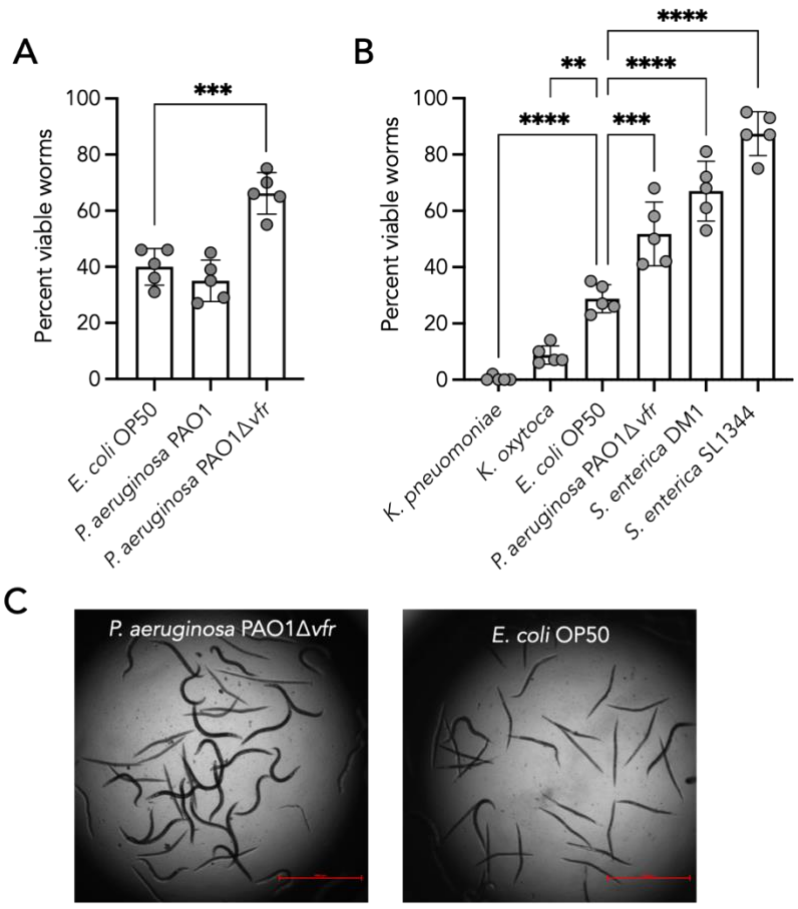

Figure 5: Exposure to pathogens during development alters *C. elegans* survival in the *P. aeruginosa* liquid killing assay. A. Pre-exposure to PAO1 harbouring a deletion of *vfr* can protect animals from subsequent PA14 infection. **B.** Pre-exposure to Gram-negative pathogens differentially impacts survival in the liquid killing assay. Animals were pre-treated with strains listed on the x-axis, then exposed to PA14 in the liquid killing assay. Each data point represents a well containing approximately 50 animals (A & B). Asterisks indicate strains that were significantly different from OP50 by one-way ANOVA with Dunnett's multiple comparisons test. **** p<0.0001; ***p<0.001; **p<0.01 (A & B). **C.** Pre-exposure to PAO1Δ*vfr* increases survival in the liquid killing assay. The strain listed on the top of

the image was used to pre-treat animals prior to exposure to PA14 in the liquid killing assay. Scale = 1mm.

Given the observation that pre-treatment with mildly virulent strains can confer protection from subsequent infection with highly virulent PA14, we sought to determine if other Gram-negative pathogens could elicit protection, under the hypothesis that exposure to mildly virulent pathogens may 'prime' the worm's immune system for a more effective response when later challenged with infection. Although *C. elegans* lack specialized immune cells, and therefore do not produce cell-mediated immunological memory, however, previous studies have described 'pathogenic learning' in *C. elegans* (Moore, Kaletsky, and Murphy 2019; Y. Zhang, Lu, and Bargmann 2005). When animals are exposed to PA14, they will avoid the pathogen more rapidly upon subsequent exposure compared to animals that have not been previously exposed (Moore, Kaletsky, and Murphy 2019). This aversive behaviour is induced by olfactory cues produced by pathogenic bacteria (Y. Zhang, Lu, and Bargmann 2005). However, survival advantages attributed to aversive pathogenic learning have only been associated with an enhanced ability to rapidly avoid the pathogen, reducing infection-mediated damage that would otherwise shorten lifespan (Moore, Kaletsky, and Murphy 2019). Avoidance is not possible in the liquid killing assay, indicating a separate mechanism underlies this protective effect. Pre-treatment with other pathogens will aid in understanding the specificity

of this phenomenon, as there are both broad-spectrum and pathogen-specific responses initiated by *C. elegans'* innate immune system (Pukkila-Worley and Ausubel 2012).

Worms were pre-treated with wild-type *Klebsiella pneumoniae*, *K. oxytoca*, and two strains of *Salmonella* Typhimurium prior to exposure to PA14 in the liquid killing assay. Although these strains all reduce the adult lifespan of *C. elegans* **(Table 3)**, in contrast to highly pathogenic PA14, *C. elegans* larvae exhibit developmental rate comparable to animals grown on OP50 when feeding on lawns each of these pathogens. The two species of *Klebsiella* decreased viability in the liquid killing assay compared to OP50 pre-treated animals, whereas the two strains of *S*. Typhimurium provided a significant survival advantage.

Table 3. Pathogenicity of bacterial strains used in this study

Strain	Source	*glp-4* LT50 on agar	Reference
P. aeruginosa PA14	Type strain, Burrows Lab	5 days	Mercedes DiBernardo, MacNeil Lab
P. aeruginosa PAO1	Type strain, Burrows Lab	7 days	Mercedes DiBernardo, MacNeil Lab
P. aeruginosa PAO1Δ*vfr*	Type strain mutant, Burrows Lab	8 days	Mercedes DiBernardo, MacNeil Lab
K. pneumoniae 32D9	Clinical isolate, Surette Lab	8.5 days	Kim Pho, MacNeil Lab
K. oxytoca 31D1	Clinical isolate, Surette Lab	N/A	N/A
S. Typhimurium SL440	Type strain, Coombes Lab	5 days	TeKippe and Aballay, 2010. PLoS One. (Tekippe and Aballay 2010)

S. Typhimurium DM1	Clinical isolate, Coombes Lab	N/A	N/A

3.3 Resistance to liquid killing is not passed onto progeny and occurs independent of intestinal colonization

The ability to pass on 'memory' of infection to naïve progeny is an established phenomenon in *C. elegans.* Learned PA14 avoidance is transgenerational and can be maintained for four generations after exposure. We first asked if protection from pre-exposure could be passed on intergenerationally. If so, this could focus mechanistic studies on known pathways that mediate inherited phenotypes. When the parental generation was raised on OP50 and the offspring on PAO1 Δ*vfr*, the expected protection from liquid killing was observed compared to animals with no previous exposure to PAO1 Δ*vfr*, but when only the parental generation was exposed to PAO1 Δ*vfr*, this protective phenotype was lost **(Fig 6A).** Surprisingly, when the same experiment was performed with *S. enterica* SL1344 pre-exposure, offspring whose parents were exposed to SL1344 exhibited significantly reduced survival in the liquid killing assay compared to animals only exposed to OP50 in both generations **(Fig 6B).** This is in contrast to the protective effect observed when animals were grown on SL1344 prior to exposure to PA14 in the same generation. As *P. aeruginosa* and *S. enterica* mediated protection from liquid killing was not passed onto progeny, this was not further investigated.

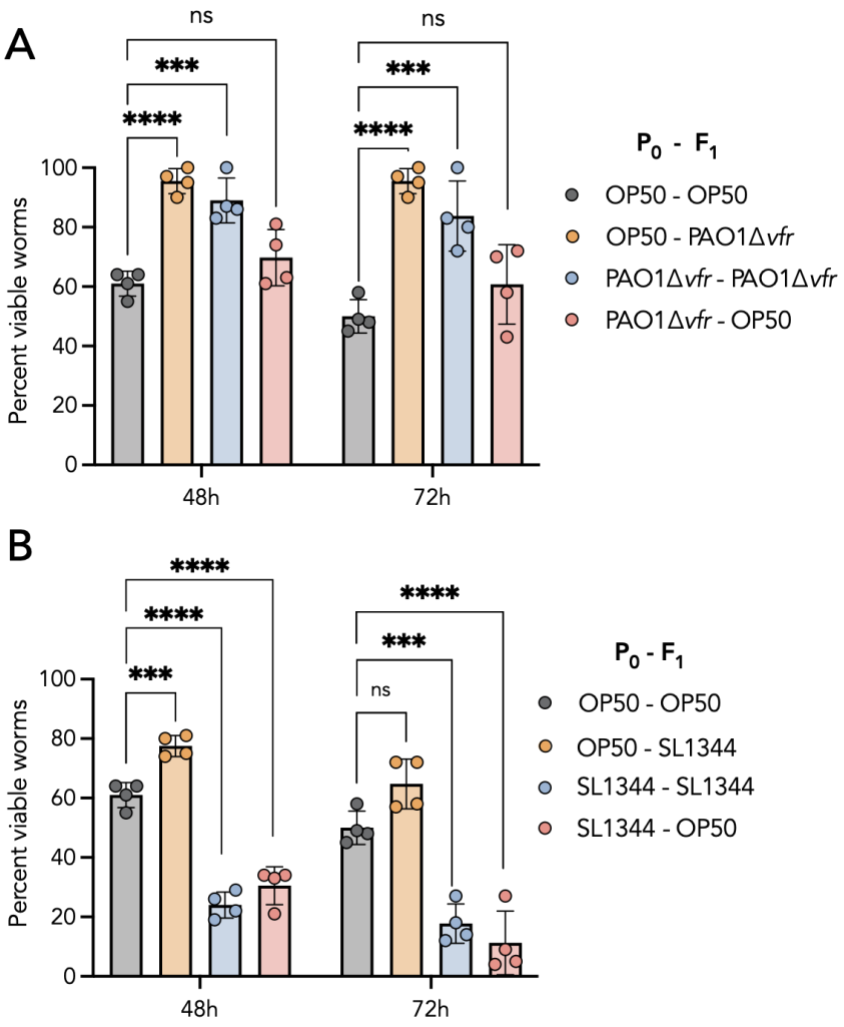

Figure 6: Pathogen-induced protection from liquid killing is not inherited. A. Naïve progeny whose parents were grown on PAO1 Δ*vfr* ~~do not exhibit~~ lack enhanced resistance to infection. **B.** Naïve progeny whose parents were grown on SL1344 exhibit reduced survival during to liquid killing, compared to animals grown on OP50 in both generations, then exposed to PA14 in the liquid killing assay. Each data point represents a well containing approximately 50 animals. Asterisks indicate strains that were significantly different from OP50-OP50 treatment by one-way ANOVA with Dunnett's multiple comparisons test. ****p<0.0001; ***p<0.001.

Since the protective phenotype was not heritable, we considered mechanisms of infection resistance that would affect only the population exposed to the protective diet. Colonization resistance refers to the prevention of pathogen growth via commensal microbes pre-emptively occupying the niche required for infection (Sorbara and Pamer 2018). Although *P. aeruginosa* and *S. enterica* are not native *C. elegans* commensals, they may competitively exclude growth of PA14 in the intestine and prevent infection-mediated damage during liquid killing, ultimately leading to increased survival. Because both pathogens can colonize the *C. elegans* intestine, it is plausible that they are preventing PA14 colonization during infection (Desai et al. 2019; M.-W. Tan, Mahajan-Miklos, and Ausubel 1999). Conversely, exposure to these pathogens during the pre-treatment period could activate innate immune pathways and lead to the production of effector molecules such as lysozymes or antimicrobial peptides that would enhance clearance of PA14 during liquid killing.

To understand if pre-treatment was decreasing pathogen load in the gut, animals fed each protective diet were exposed to PA14 tagged with mCherry (PA14 + pBADGr-*pilE::mCherry*) in the liquid killing assay, and fluorescence signal was compared to animals only fed OP50 during development. First, to ensure that the fluorescent construct did not alter

pathogenicity, survival outcomes were compared to that of WT PA14. The fluorescent construct did not alter survival outcomes in the liquid killing assay **(Fig 7E).**

Pre-treatment with PAO1 Δ*vfr* nor SL1344 did not alter PA14 load in the intestine during liquid killing, as visualized by similar degrees of fluorescence intensity regardless of the pre-treatment condition **(Fig 7B-D).** Images were taken 24 hpi to ensure the majority of animals were still alive upon imaging; dead animals were omitted from analysis. To ensure that the visualized fluorescence was representative of the microbial load in the gut, animals from the liquid killing assay were washed and lysed to determine viable colony counts. CFU counts from each population of pre-treated animals were not significantly different from that of animals fed only OP50 during development **(Fig 7F).** This suggests that the protective effects of pre-treatment are not mediated by improved clearance of PA14 cells from the intestine during infection.

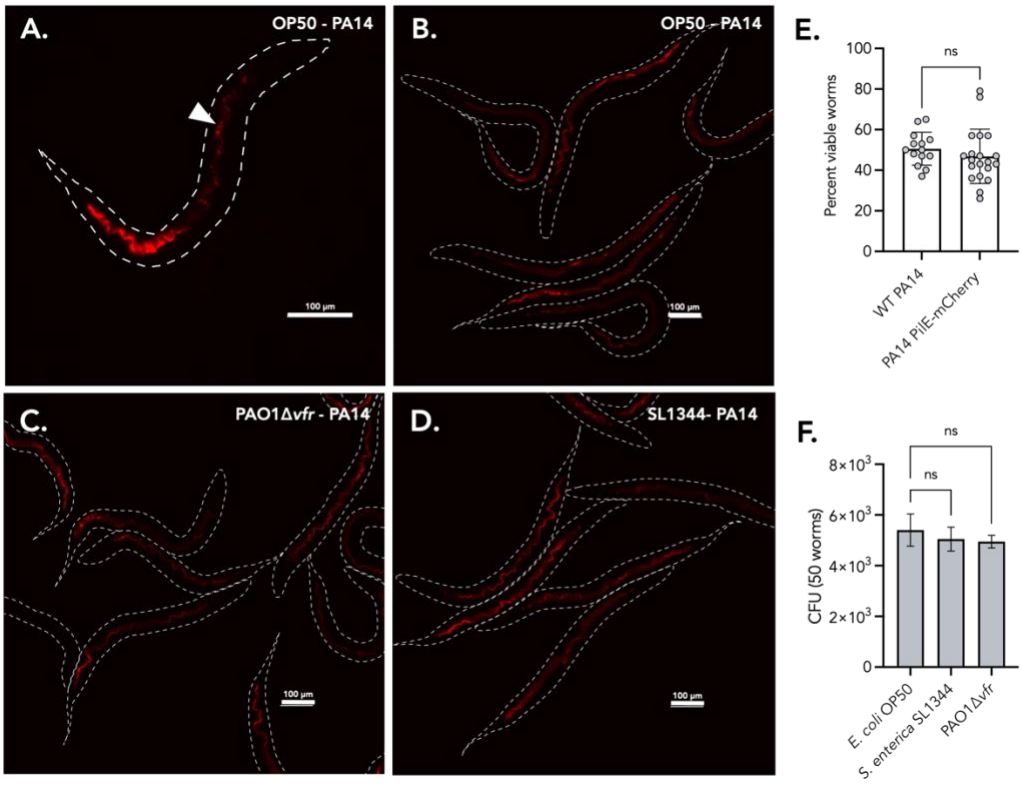

Figure 7: Pre-exposure to pathogens does not prevent colonization of the intestine by PA14-mCherry in the liquid killing assay. **A – D.** Animals were imaged 24h post-infection in the liquid killing assay, following pre-exposure to either *E. coli* OP50, *P. aeruginosa* PAO1∆vfr or *S. enterica* SL1344. The white arrow denotes a cluster of intact fluorescent PA14 cells. Scale = 100µm. **E.** Presence of the *pilE::mCherry* construct does not impact PA14 pathogenicity in the liquid killing assay. Statistical significance was determined using an unpaired t-test. Each data point represents a well containing approximately 50 animals. **F.** Viable intestinal colony counts from the liquid killing assay are not altered by protective pre-treatment by One-Way ANOVA with Dunnett's multiple comparisons test.

3.4 Pre-exposure to *P. aeruginosa*, but not *S. enterica*, mediates protection from slow killing

Given *C. elegans* mounts distinct responses to *P. aeruginosa* infection in liquid and agar-based killing conditions (Kirienko et al. 2013; M.-W. Tan, Mahajan-Miklos, and Ausubel 1999), we aimed to determine if pre-exposure to *P. aeruginosa* or *S. enterica* could also elicit protection in the agar-based slow killing assay. Pre-treatment with PAO1Δ*vfr* was able to significantly delay the time to 50% death during slow killing, however, pre-treatment with SL1344 did not **(Fig 8A and B)**. Neither strain altered survival of animals on OP50 lawns **(Fig 8A)**. These results indicate distinct mechanisms of protection in response to the two different species.

To determine if the protective factor(s) produced by either *P. aeruginosa* or *S. enterica* were secreted, the supernatants of each strain were sterilized and added to lawns of OP50. Animals were pre-treated on supernatant-supplemented lawns, then exposed to PA14 in the liquid killing assay. Neither the supernatants collected from microbiotal *P. aeruginosa* isolates, nor the supernatant from *S. enterica* SL1344 induced protection from PA14 liquid killing **(Fig 8C & D)**. Interestingly, animals pre-exposed to lawns of OP50 supplemented with *K. pneumoniae* supernatant exhibited a slight, but not significant increase in survival compared to lawns of OP50 alone, whereas live lawns of *K. pneumoniae* result in increased death in the liquid

killing assay. Together, this suggests that the factor(s) underlying protection from, or synergy with, PA14 infection are not secreted. Whether the bacterial cells must be viable or intact to induce protection remains to be investigated.

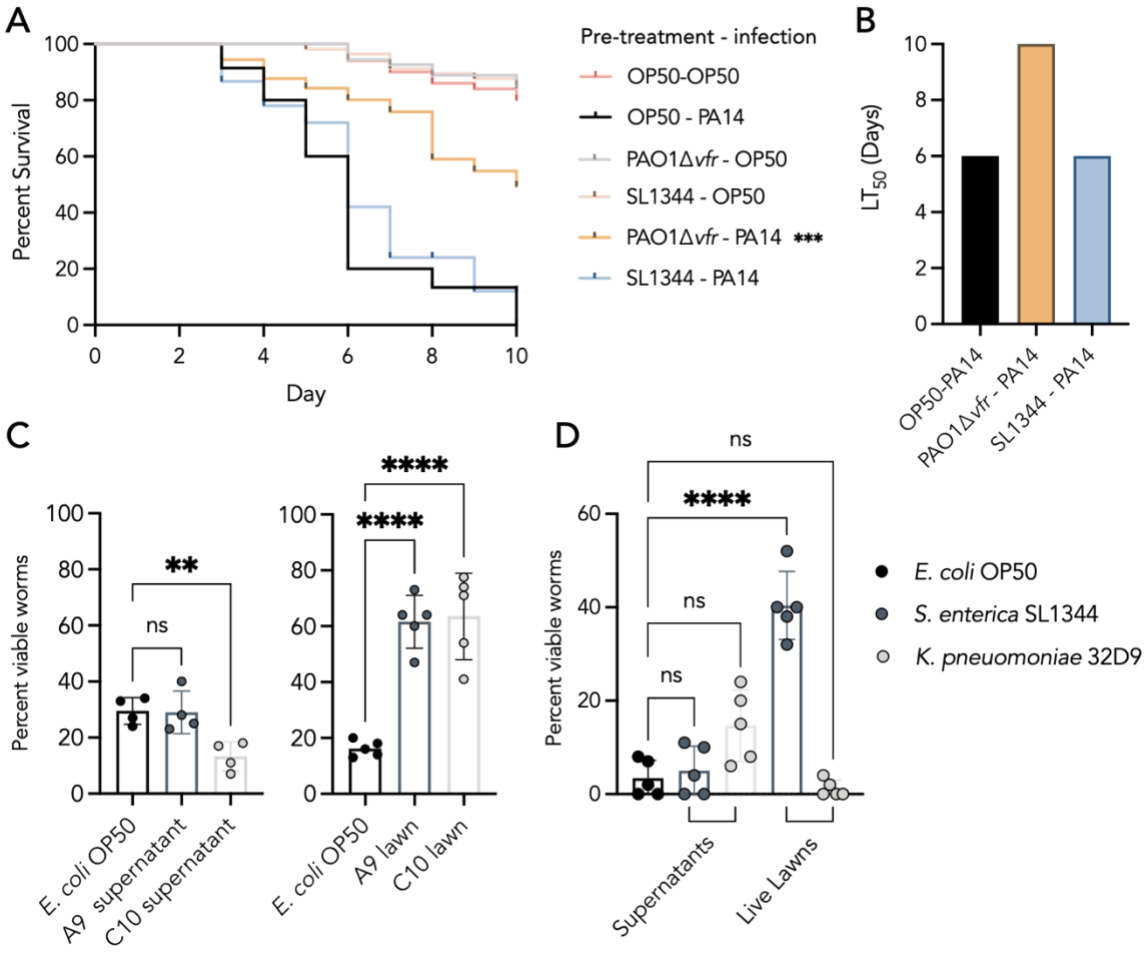

Figure 8. Pre-exposure to *P. aeruginosa*, not *S. enterica*, mediates protection from slow killing. A. Animals pre-exposed to PAO1 Δ*vfr* exhibit increased lifespan on PA14 lawns but not on OP50. SL1344 pre-exposure does not increase lifespan on PA14 or OP50. Asterisks indicate treatment conditions significantly different than OP50 - PA14 by Gehan-Breslow-

Wilcoxon test. **B**. Pre-exposure to PAO1Δ*vfr* increases LT_{50} of *glp4(bn2)* worms on PA14 from six to ten days. **C & D**. OP50 lawns supplemented with supernatants of clinical *P. aeruginosa* isolates identified from the microbiota isolate library screen or *S. enterica* SL134 do not protect from liquid killing. *K. pneumoniae* supernatants are not detrimental to survival during liquid killing, whereas live lawns are. Each data point represents a well containing approximately 50 animals. Asterisks indicate strains that were significantly different from OP50-OP50 treatment by one-way ANOVA with Dunnett's multiple comparisons test. ****p<0.0001; ***p<0.001; **p<0.01.

3.5 Protective pre-treatment alters expression of a *P. aeruginosa*-specific infection response gene

The defense mechanisms involved in the host response to *P. aeruginosa* include conserved innate immune and stress response pathways, as summarized in Figure 1. Transcriptional profiling during infection suggested that *P. aeruginosa* in liquid killing conditions initiates a distinct response compared to *P. aeruginosa* infection on solid plates (Kirienko, Ausubel, and Ruvkun 2015; Tjahjono and Kirienko 2017). *C. elegans* response to infection in the liquid assay employs factors previously attributed to abiotic stressors, and includes regulators of autophagy, mitophagy, and the hypoxia response (Tjahjono and Kirienko 2017). The bZIP family transcription factor ZIP-2 induces expression of a subset of genes responsive to mitochondrial stress, as well as a number of genes responsive

to *P. aeruginosa* on solid media (Reddy et al. 2016). As such, *zip-2* mutants have increased sensitivity to *P. aeruginosa* in both solid and liquid media (Estes et al. 2010; Tjahjono and Kirienko 2017). Given the pre-treatment period occurs on solid plates and the infection occurs in liquid conditions, and the importance of ZIP-2-regulated genes in both conditions, we made use of a *C. elegans* strain harbouring a transcriptional reporter of a ZIP-2 regulated gene, *irg-1* (infection response gene 1) (Estes et al. 2010). This gene is regulated by ZIP-2, independent of PMK-1 and other broad-spectrum pathogen response pathways, and is specifically induced by virulent *P. aeruginosa*, not other bacterial pathogens or non-pathogenic mutants of PA14 (Estes et al. 2010). Characterization of the *irg-1p*::*GFP* reporter under our assay conditions was hypothesized to show whether pre-treatment with protective strains affects a major defense-response pathway involved in *P. aeruginosa* infection.

First, expression of *irg-1p::GFP* was characterized in the liquid killing assay, because this reporter has previously been imaged exclusively in slow killing conditions in solid media. On solid media, *irg-1p::GFP* expression is confined to the intestine, whereas in liquid, there is a distinct induction of expression in the pharynx as well **(Fig 9A,B,F)** (Dunbar et al. 2012a; Estes et al. 2010; Reddy et al. 2016). Although there was variability in the expression of *irg-1p::GFP* among PA14-exposed animals, we observed an

approximate three-fold increase in fluorescence intensity compared to animals exposed to OP50 in liquid **(Fig 9E).** Expression of *irg-1p::GFP* during PA14 liquid killing was also visualized in animals pre-treated with PAO1 Δ*vfr* and SL1344. When animals were pre-treated with PAO1 Δ*vfr* prior to exposure to PA14, *irg-1p::GFP* expression was decreased in both the intestine and the pharynx compared to animals pre-treated with OP50. Conversely, when animals were pre-treated with SL1344, intestinal expression of *irg-1p::GFP* was significantly increased and pharyngeal expression was decreased. **(Fig 9B-F).** Both protective diets decreased expression in the pharynx, which appears unique to liquid killing conditions, whereas *P. aeruginosa* pre-treatment decreased *irg-1p::GFP* expression overall and *S. enterica* pre-treatment increased it in the intestine. The significance of spatial differences in *irg-1p::GFP* expression is not well-understood, as the function of *irg-1* in the immune response has not yet been elucidated. To determine if *irg-1* plays a protective role in liquid killing, *irg-1* was knocked down via RNAi. Compared to empty vector (L4440) control, *irg-1* knockdown sensitized *C. elegans* to PA14 liquid killing **(Fig 9G).** *zip-2* knockdown was used as a positive control, as *zip-2* null mutants exhibit increased sensitivity to both solid and liquid killing **(Fig 9F)** (Kirienko, Ausubel, and Ruvkun 2015)**.**

If *irg-1* knockdown sensitizes animals to liquid killing, its down-regulation in the liquid assay upon protective pre-treatment appears counter-intuitive. Furthermore, pre-treatment with *P. aeruginosa* and *S. enterica* prior to liquid killing has opposing effects on the intestinal expression of *irg-1p::GFP.* To further understand the role *irg-1* may play in *P. aeruginosa*/*S. enterica*-induced protection from infection, its function must be abrogated during the pre-treatment period. However, since RNAi is achieved via feeding *C. elegans* dsRNA-expressing *E. coli* during development, and our assays also require pre-treatment with the protective strains during development, a causal relationship between alteration of *irg-1* expression and *P. aeruginosa*/*S. enterica*-induced protection cannot currently be explored, due to this technical limitation. Construction of a *C. elegans* strain lacking a functional copy of *irg-1* is one way to circumvent this limitation.

A decrease in *irg-1* expression could be the result of protective effects of pre-treatment rather than a causal factor. The extent of *irg-1* induction is correlated with virulence, as pathogenic *P. aeruginosa* isolates such as PA14 are able to up-regulate its expression, whereas weakly pathogenic isolates are not (Estes et al. 2010). Additionally, *irg-1* is up-regulated by agents that inhibit translation, as well as the heavy metal cadmium, suggesting *irg-1* likely responds to the disruption of specific cellular processes (Estes et al. 2010). This leads to the hypothesis that protective

pre-treatment is reducing the virulence of PA14 in the liquid killing assay such that the damage induced during infection is less severe, and a decrease in *irg-1p::GFP* expression reflects this.

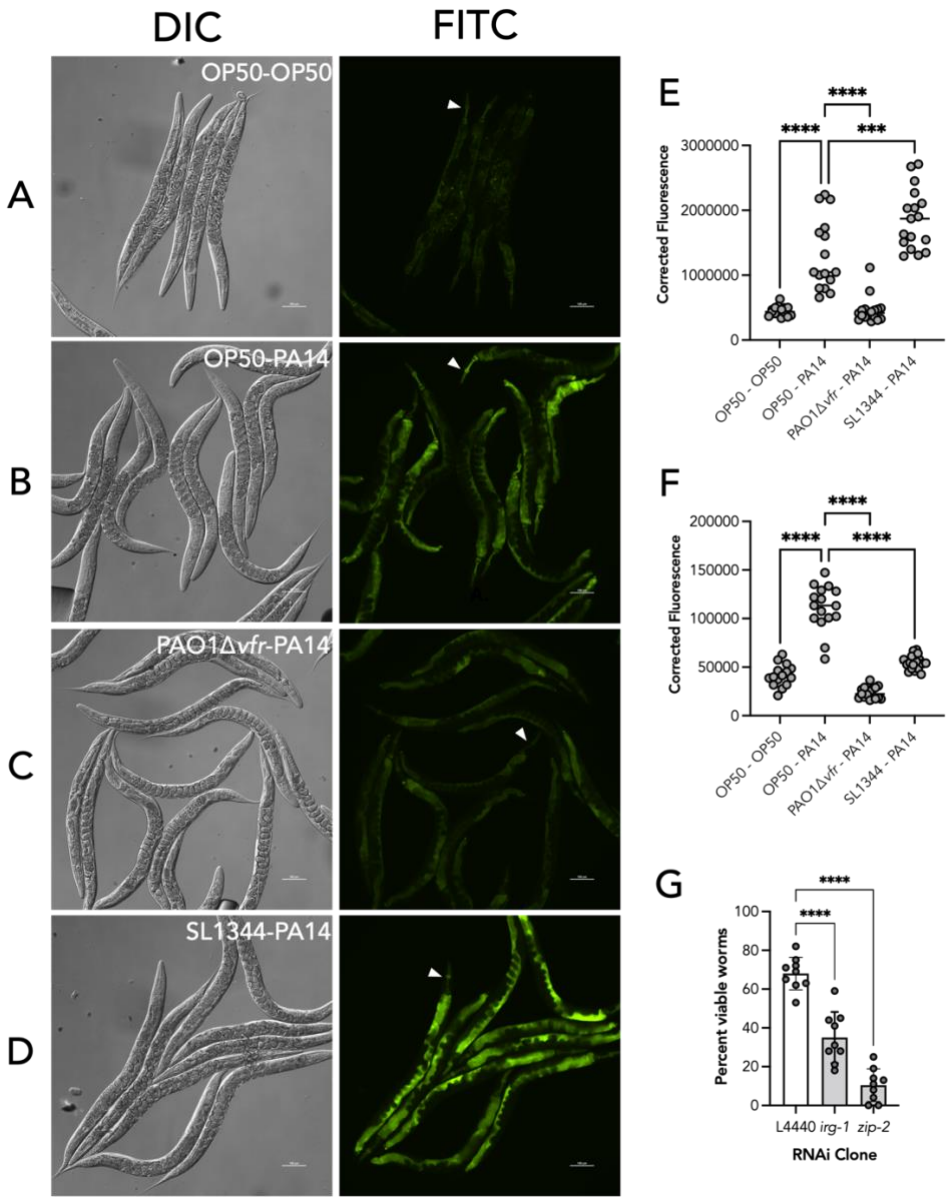

Figure 9. Pre-treatment with *P. aeruginosa* and *S. enterica* alters *irg-1p::GFP* expression during liquid killing. A – D. *irg-1p::GFP* animals were imaged 24h post-infection in the liquid killing assay. First strain

denotes the pre-exposure condition, the second denotes the liquid assay strain. White arrows denote a representative pharynx. **E – F.** ImageJ was used to quantify *irg-1p::GFP* fluorescence intensity. Each data point represents a single animal. **G**. Knockdown of *irg-1* by RNAi sensitizes animals to liquid killing. Each data point represents a well containing approximately 50 animals. Asterisks indicate strains that were significantly different from OP50-OP50 treatment by one-way ANOVA with Dunnett's multiple comparisons test. ****p<0.0001; ***p<0.001.

3.6 Vitamin B12 protects from liquid killing and alters *irg-1p::GFP* expression.

Previous work has shown that bacterially-produced vitamin B12 has multiple effects on *C. elegans* physiology, including acceleration of development, reduction in fertility, and increased resistance to abiotic stressors and infection by pathogenic bacteria (MacNeil et al. 2013; Revtovich, Lee, and Kirienko 2019; Watson et al. 2014). Like humans and all other animals, *C. elegans* cannot synthesize vitamin B12 *de novo* and therefore must obtain it from its diet (Bito et al. 2013). Revtovich *et al.* (2019) showed that a diet of *E. coli* HT115 is a more abundant source of vitamin B12 than OP50, and can protect *C. elegans* from PA14 liquid killing, as well as other stressors such as heat shock and oxidative stress (Revtovich, Lee, and Kirienko 2019). This protection is dependent on methylmalonyl-CoA mutase (MMCM-1/MUT) activity, which uses vitamin B12 as a cofactor to convert propionyl-CoA to succinyl-CoA (Revtovich, Lee, and Kirienko 2019).

The improper breakdown of short-chain fatty acids such as propionate can lead to mitochondrial dysfunction in both humans and *C. elegans* (Bito et al. 2013; R. J. Chandler et al. 2009). Increased provision of vitamin B12 by HT115 reduces propionyl-CoA toxicity via conversion to succinyl-CoA by MMCM-1, resulting in improved mitochondrial homeostasis (Revtovich, Lee, and Kirienko 2019). This overall improvement in mitochondrial health resulted in reduced susceptibility to both infection and abiotic stressors (Revtovich, Lee, and Kirienko 2019).

Given that species within the genus *Pseudomonas* can synthesis vitamin B12 *de novo*, it is possible that protection from PA14 infection by non-pathogenic strains of *P. aeruginosa* may be due to enhanced provision of vitamin B12 (Crespo, Blanco-Cabra, and Torrents 2018; Fang, Kang, and Zhang 2017). To test this hypothesis, we generated a PAO1 strain with reduced virulence (PAO1 Δ*vfr*) and deficient in aerobic vitamin B12 production by deleting *cobN*, a gene involved in early cobalt insertion in the aerobic branch of B12 biosynthesis in *Pseudomonas* species (Crespo, Blanco-Cabra, and Torrents 2018). To confirm that this mutation effectively decreases vitamin B12 availability to *C. elegans*, a *C. elegans* reporter strain, *acdh-1p::GFP*, was used. *acdh-1* encodes an acyl-CoA dehydrogenase that is responsive to propionate accumulation (Gilst, Hadjivassiliou, and Yamamoto 2005; Watson et al. 2013, 2014). Given

vitamin B12 is a cofactor for MMCM-1, the enzyme responsible for conversion of propionyl-CoA to succinyl-CoA, this reporter can be used as a proxy for vitamin B12 availability. When ample dietary B12 is provided its expression is repressed; this can be achieved by direct addition of vitamin B12 to the growth media, or by B12-producing bacterial diets, such as species of *Pseudomonas* or *Comamonas* (Watson et al. 2014).

E. coli OP50 is a poor source of vitamin B12, so worms reared on OP50 actively express GFP under control of the *acdh-1* promoter **(Fig 10A,G)** (Watson et al. 2014). As expected, a diet of PAO1 Δ*vfr* repressed activation of the *acdh-1* promoter, whereas a diet of PAO1 Δ*vfr* Δ*cobN* resulted in its activation **(Fig 10E-G).** Although *acdh-1p::GFP* expression was much higher in OP50-fed animals than animals fed PAO1 Δ*vfr* Δ*cobN*, deletion of *cobN* increased expression of the reporter compared to PAO1 Δ*vfr*, suggesting that deletion of *cobN* interrupts aerobic B12 biosynthesis and reduces B12 bioavailability **(Fig 10A, E-G).** Although deletion of *cobN* failed to increase *acdh-1p::GFP* expression relative to OP50, the increase in fluorescence intensity observed when animals were fed the *cobN* mutant indicates they are more starved for B12 than animals fed PAO1 Δ*vfr*. This reduction in B12 availability did not cause a significant reduction in survival during liquid killing when PAO1 Δ*vfr* Δ*cobN* was used to pre-treat animals prior to exposure to PA14 in the liquid killing assay **(Fig 10H).** PAO1 Δ*vf*

*r*Δ*cobN* pre-treatment remained significantly protective compared to OP50 pre-treatment, consistent with significantly lower *acdh-1p::GFP* expression in PAO1 Δ*vfr* Δ*cobN*-fed animals. This result suggests that although biosynthesis contributes to the overall amount of B12 provided by *P. aeruginosa*, it may also exhibit an enhanced ability to import vitamin B12 from the surrounding media compared to OP50.

S. enterica is not capable of aerobic vitamin B12 biosynthesis, however, it still may contain increased levels of intracellular vitamin B12 compared to OP50, like PAO1 Δ*vfr* Δ*cobN* (Jeter, Olivera, and Roth 1984). Reporter animals were fed *S. enterica* SL1344, as well as a clinical isolate of *K. pneumoniae* to determine if it is also a poor source of vitamin B12, given pre-treatment with this species causes decreased survival in the liquid killing assay **(Fig 10)**.

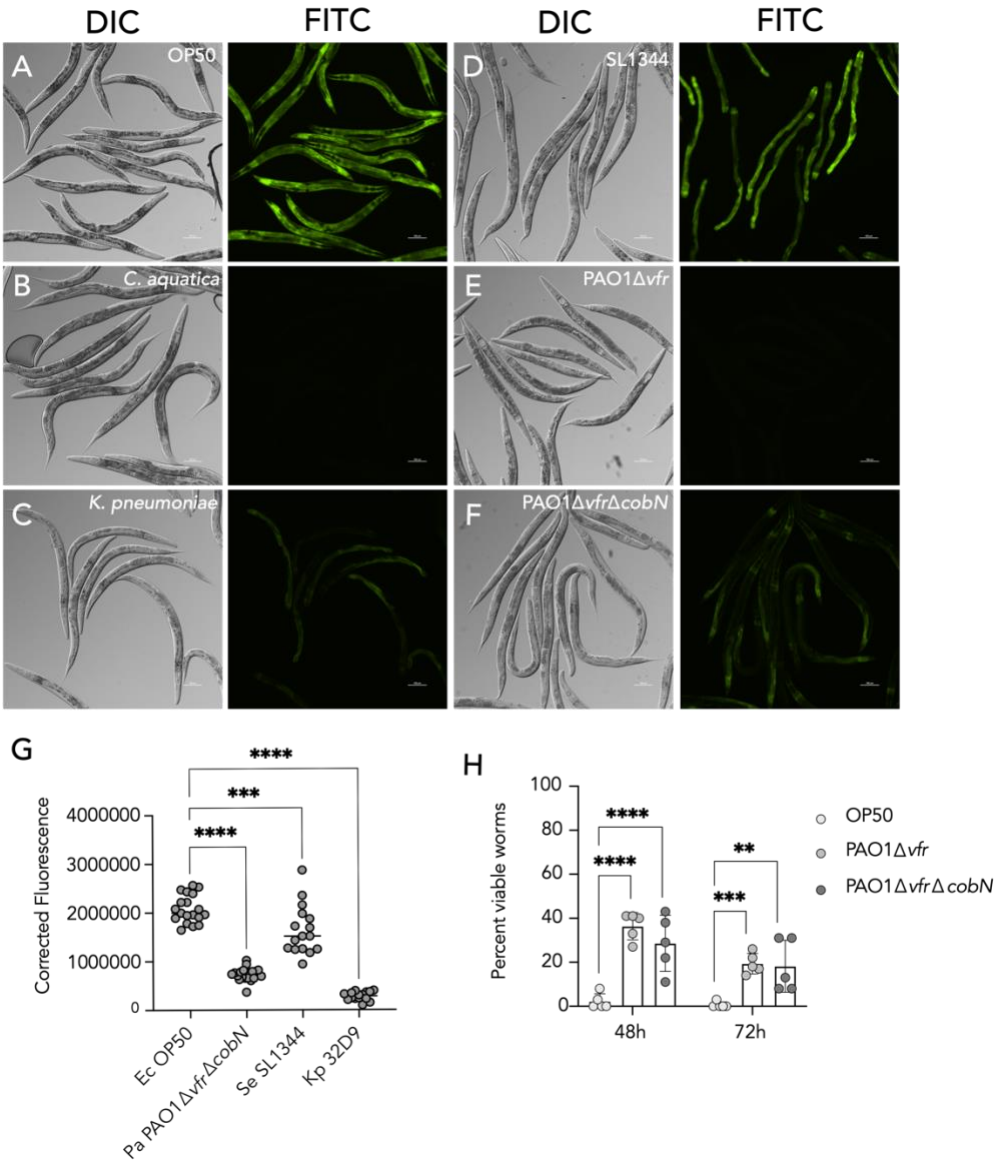

Figure 10. Disruption of *Pseudomonas* vitamin B12 biosynthesis does not impact protection from PA14 infection. A – F. Deletion of *cobN* in *Pseudomonas* does not increase *acdh-1p::GFP* expression to the same degree as *E. coli* OP50. L1s were transferred to lawns of each bacterial diet and imaged after 42h, at the late L4 stage. **G.** ImageJ was used to quantify *acdh-1p::GFP* fluorescence intensity. Each data point represents a single animal. **H**. Pre-treatment with PAO1Δ*vfr*Δ*cobN* results in a slight but insignificant decrease in survival compared to pre-treatment with parent

strain PAO1Δ*vfr*. Each data point represents a well containing approximately 50 animals. Asterisks indicate strains that were significantly different from OP50-OP50 treatment by one-way ANOVA with Dunnett's multiple comparisons test. ****p<0.0001; ***p<0.001.

When *acdh-1p::GFP* reporter animals were fed SL1344, fluorescence intensity was slightly lower than OP50-fed animals **(Fig 10A,D,G)**. *acdh-1p::GFP* expression was confined to the intestine, whereas with OP50 it was also expressed in the body wall muscle and the hypodermis **(Fig 10A,D)**. *acdh-1p::GFP* expression was significantly lower in animals fed *K. pneumoniae* versus OP50, indicating that although *K. pneumoniae* is detrimental to survival in the liquid killing assay, it is a far more abundant source of B12 than OP50. Taken together, induction of *acdh-1p::GFP* expression does not correlate with the ability to protect *C. elegans* from liquid killing, suggesting that while provision of vitamin B12 by the bacterial diet may play a partial role in protection from infection, additional protective or synergistic determinants remain to be uncovered.

Given the alteration of *irg-1* expression caused by pre-exposure to *P. aeruginosa* and *S. enterica*, we aimed to determine if vitamin B12 alone influences *irg-1* expression. *irg-1p::GFP* animals were fed OP50 cultured on media supplemented with vitamin B12, then transferred to the liquid killing assay. Similar to what was observed with *P. aeruginosa* pre-exposure,

vitamin B12 supplementation decreased *irg-1p::GFP* fluorescence intensity in the intestine as well as the pharynx **(Fig 11).** *irg-1p::GFP* expression did not further decrease with increasing vitamin B12 concentration, indicating the lowest concentration tested (0.05 µg/ml) was sufficient to induce this phenotype **(Fig 11)**. Although this does not implicate B12 as the sole protective factor in *P. aeruginosa* pre-exposure, it does align with the body of evidence that *irg-1* responds to infection-mediated damage, rather than the pathogenic bacterium itself (Dunbar et al. 2012a; McEwan, Kirienko, and Ausubel 2012). Previous studies have reported that *irg-1p::GFP* is up-regulated upon knockdown of enzymes that act within the mitochondria, so it is reasonable that addition of vitamin B12, which improves mitochondrial homeostasis, would cause a reduction in *irg-1p::GFP* expression (Dunbar et al. 2012b; Revtovich, Lee, and Kirienko 2019; Y et al. 2014).

To further explore the role vitamin B12 may play in *P. aeruginosa* and *S. enterica*-induced protection from infection, we aimed to reduce the amount of vitamin B12 present in the growth media, to control for differences in ability to import vitamin B12 from the extracellular environment. Gram-negative bacteria import B12 and other vitamins using TonB-dependent transporters (TBDTs), which may vary in abundance in the outer membrane of Gram-negative species (Noinaj et al. 2010). To generate media with very low amounts of vitamin B12, soy-peptone was used (Watson et al. 2016).

However, when L1s were cultured on lawns of PAO1 Δ*vfr* grown on soy-peptone NGM, larval development was stunted **(Fig 11C)**. Although SL1344 grown on soy-peptone NGM did not slow larval development to the same extent as PAO1 Δ*vfr*, the population contained an increased number of younger animals relative to populations cultured on OP50 under the same conditions **(Fig 12B)**. Because ensuring all animals are the same age is essential to control for age-related differences in survival, these populations were not tested in the liquid killing assay.

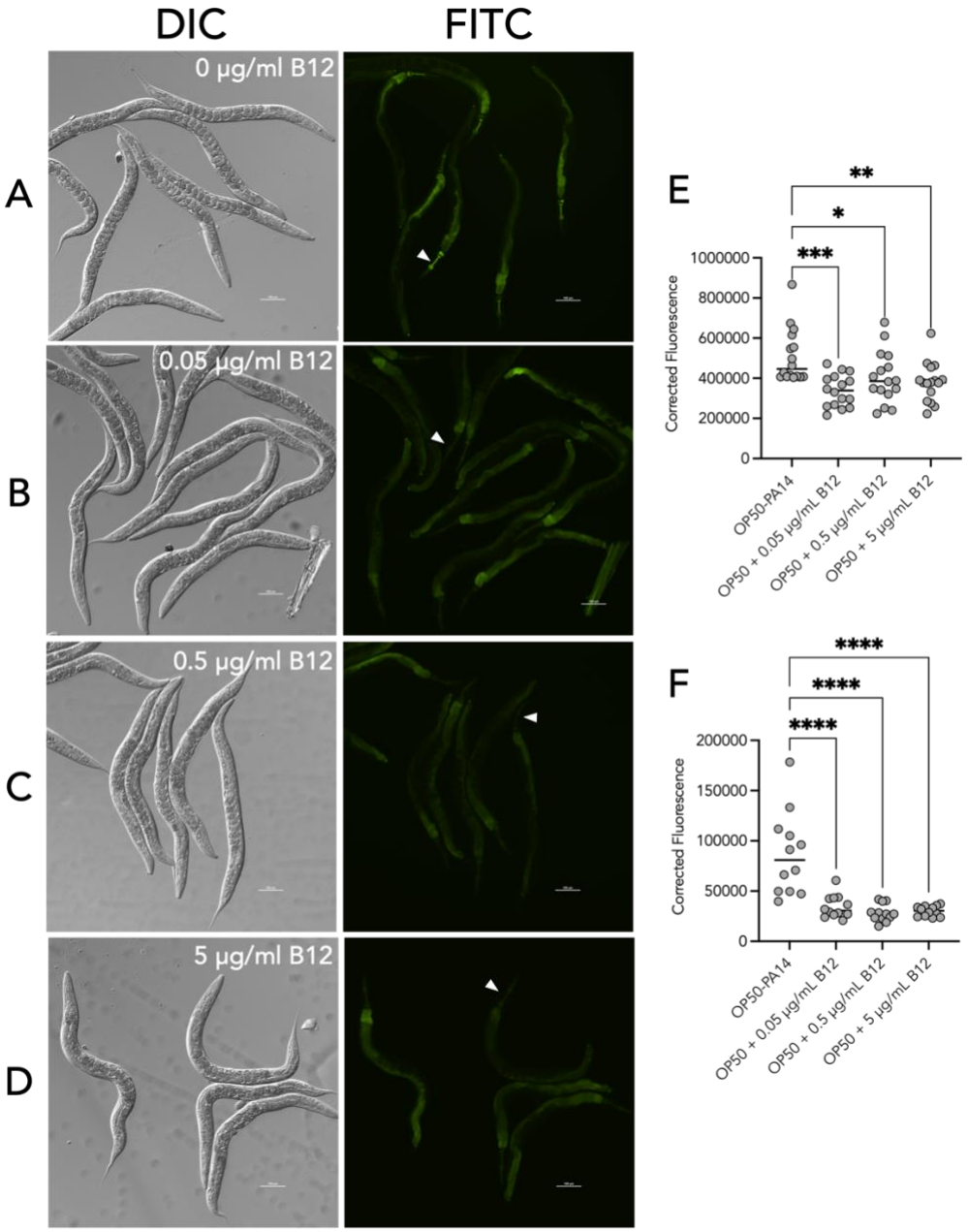

Figure 11. Vitamin B12 alters *irg-1p::GFP* expression. A – D. *irg-1p::GFP* reporter animals were imaged 24 h post-infection in the liquid killing assay, following pre-exposure to OP50 grown on media supplemented with increasing concentrations of vitamin B12. White arrows denote a representative pharynx **E – F.** ImageJ was used to quantify *irg-1p::GFP* fluorescence intensity. Each data point represents a single animal. **E.**

Measurements were taken of the whole body of the animal. **F**. Measurement were confined to the pharynx. Asterisks indicate strains that were significantly different from OP50-OP50 treatment by one-way ANOVA with Dunnett's multiple comparisons test. ****p<0.0001; ***p<0.001; **p<0.01; *p<0.1.

Vitamin B12-mediated protection from infection is dependent on MMCM-1, an enzyme that uses vitamin B12 as a cofactor and functions in the methylmalonyl/succinyl-CoA breakdown pathway. To determine if MMCM-1 is essential for *P. aeruginosa* and *S. enterica*-induced protection from infection, we introduced a null *mmcm-1(ok1637)* allele into the *glp-4(bn2)* background. When this strain was pre-treated with either PAO1 Δ*vfr* or SL1344, survival in the liquid killing assay was still increased compared to OP50 pre-treatment, though to a lesser degree than *glp-4* animals with functional MMCM-1, suggesting that MMCM-1 function is only partially responsible for the protective phenotypes observed **(Fig 12A).** These data suggest that other vitamin B12-independent pathway(s) are involved in mediating *P. aeruginosa* and *S. enterica*-induced pathogen resistance.

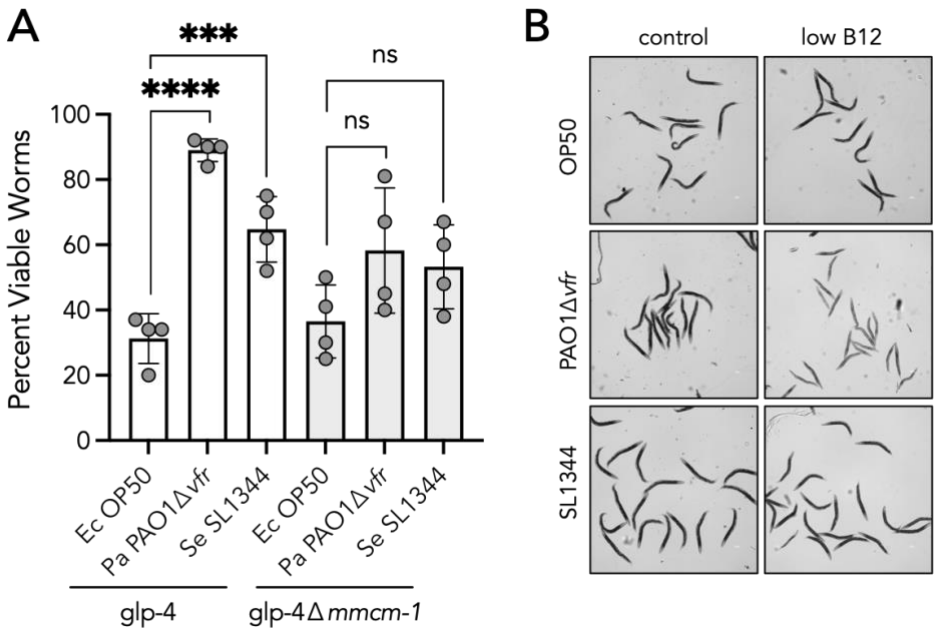

Figure 12. MMCM-1 partially mediates protection from infection. A. Pre-exposure to *P. aeruginosa* and *S. enterica* still protects animals with a *mmcm-1* null allele from liquid killing, but to a lesser degree than wild-type. Each data point represents a well containing approximately 50 animals. Asterisks indicate strains that were significantly different from OP50 pre-treatment by one-way ANOVA with Dunnett's multiple comparisons test. ****p<0.0001; ***p<0.001. **B**. Pathogenic lawns grown on low-B12 media cause slowed larval development.

CHAPTER 4. DISCUSSION

4.1 Protective pre-exposure reduces infection-mediated damage in the liquid killing assay

While screening a library of microbiotal bacteria for isolates able to protect *C. elegans* from *P. aeruginosa* infection, we discovered that mildly pathogenic strains of *P. aeruginosa* can protect from future infection with a virulent strain of *P. aeruginosa*, PA14. Previous studies identified bacterial species able to protect *C. elegans* from infection by stimulating the innate immune response (Kissoyan et al. 2019; Montalvo-Katz et al. 2013; Rangan et al. 2016). For example, pre-exposure to both pathogenic and commensal strains of *Enterococcus faecium* can prolong survival during *Salmonella enterica* infection. This protective effect was attributed to a secreted peptidoglycan hydrolase produced by *E. faecium*, that generates fragments of peptidoglycan that activate TOL-1 mediated immunity required for resistance to *Salmonella* infection (Rangan et al. 2016; Tenor and Aballay 2008).

Commensal strains of *Pseudomonas* isolated from the natural *C. elegans* microbiome protect against both *Bacillus thuringiensis* and *P. aeruginosa* PA14 infection (Kissoyan et al. 2019; Montalvo-Katz et al. 2013). Protection from PA14 infection by a non-pathogenic strain of *P. mendocina* was mapped to the p38 MAPK pathway, indicating that pre-exposure to

Pseudomonas species primes the worm immune system for a more effective response upon subsequent pathogenic challenge. Similar to our findings, this study also showed that a PA14 *gacA* mutant with attenuated virulence protected from WT PA14 infection in the slow killing assay (Montalvo-Katz et al. 2013; M. W. Tan et al. 1999). Montalvo-Katz *et al.* (2013) also showed that pre-treatment with *P. mendocina* delayed PA14 colonization, by visualization of reduced PA14-GFP fluorescence in the intestine, suggesting that a 'primed' immune system may clear the infection more efficiently. To determine if pre-exposure to PAO1Δ*vfr* or SL1344 reduces colonization during infection in liquid culture, we visualized colonization using a fluorescent PA14 reporter, and quantified pathogen load by counting CFUs isolated from the intestine. We found that neither pre-exposure condition significantly decreased PA14 intestinal load, suggesting animals are becoming more resistant to the infection during pre-treatment, rather than clearing the pathogen more readily. This suggests that the mechanisms underlying protection observed in this study are distinct from what has been described for *P. mendocina* mediated protection. In contrast to slow killing, compromising the PMK-1/p38 MAPK pathway increases resistance to liquid killing (Kirienko, Ausubel, and Ruvkun 2015). Similarly, hyper-activation of PMK-1 signaling by RNAi-mediated knockdown of its inhibitory phosphatase, *vhp-1,* results in increased sensitivity to liquid killing (Kirienko, Ausubel, and Ruvkun 2015).

Therefore, it is unlikely that activation of PMK-1 signaling underlies *P. aeruginosa* or *S. enterica*-mediated protection infection in the liquid killing assay.

A transposon screen of *S. enterica* revealed that many of the virulence factors required for infection in *C. elegans* are encoded on the *Salmonella* pathogenicity island (SPI-1) and are secreted through the type III secretion system (T3SS) (Tenor et al. 2004). Of these, the SptP T3SS effector protein contributes to virulence by preventing the phosphorylation of PMK-1, and therefore inhibiting PMK-1/p38 MAPK signaling (Tenor et al. 2004). As *pmk-1* knockdown increases resistance to liquid killing, it is possible that *S. enterica* pre-treatment decreases PMK-1 signaling, and therefore increasing resistance to infection. To determine if *S. enterica*-mediated protection from liquid killing is *pmk-1*-dependent, we attempted to cross a *pmk-1(km25)* mutant into the *glp-4(bn2)* background, however, a mutant homozygous for both alleles was not successfully isolated.

Resistance to *S. enterica* infection also requires autophagy. Knockdown of evolutionary-conserved genes involved in activation of autophagy, called *atg* genes, results in increased susceptibility to *S. enterica* infection and allows for increased proliferation of bacterial cells in the intestinal lumen (Jia et al. 2009). Specifically, this relies on *bec-1* and *lgg-1,* the *C. elegans*

homologs of mammalian Beclin-1 and Atg8 (Jia et al. 2009; Niu et al. 2012; Takacs-Vellai et al. 2005). Both genes are essential for initiation of the autophagosome complex and are therefore indispensable for autophagy (Jia et al. 2009; Niu et al. 2012; Takacs-Vellai et al. 2005). Knockdown of either of these genes increases *C. elegans* sensitivity to PA14 liquid killing, suggesting that activation of autophagy is required for an effective host response in the liquid killing assay (Kirienko, Ausubel, and Ruvkun 2015). Autophagy is also essential for the *C. elegans* response to *P. aeruginosa* in the slow killing assay, as knocking down *bec-1* significantly reduces lifespan on PA14 (Zou et al. 2014). Furthermore, an increase in GFP::LGG-1 puncta, a reliable indicator of induction of autophagy, is observed upon *P. aeruginosa* infection on agar plates (Kirienko, Ausubel, and Ruvkun 2015; Zou et al. 2014). Therefore, pre-exposure to *P. aeruginosa* or *S. enterica* could prime the autophagic response and result in more effective clearance of cellular damage accumulated during liquid killing.

During *P. aeruginosa* infection, autophagy does not function to clear bacterial cells from the intestine, rather, it protects against necrosis imposed by *P. aeruginosa* during infection (Zou et al. 2014). In contrast to knockdown of *pmk-1*, knockdown of *bec-1* increases susceptibility to slow killing without increasing intestinal accumulation of PA14 (Zou et al. 2014). These data are similar to results of our liquid killing assay; pre-treatment with *P.*

aeruginosa or *S. enterica* increases survival without decreasing pathogen load in the gut. During response to pathogenic attack, *C. elegans* must employ mechanisms, such as *pmk-1*-regulated effector genes including lysozymes and antimicrobial peptides, to destroy the bacterium as well as mechanisms such as induction of autophagy to alleviate cellular damage imposed by the pathogen. This work shows that infection resistance can be achieved by modulating pathways responsible for pathogen clearance and those that reduce infection-mediated damage.

4.2 *irg-1* expression can be modulated by agents that reduce cellular damage during infection

irg-1 is up-regulated in response to damage induced by pathogenic *P. aeruginosa,* as it is responsive to disruption of cellular processes such as protein translation and mitochondrial activity (Dunbar et al. 2012a; McEwan, Kirienko, and Ausubel 2012; Reddy et al. 2016; Y et al. 2014). Here, we found that pre-exposure to *P. aeruginosa* and *S. enterica* differentially modulate *irg-1p::GFP* expression in response to PA14 in the liquid killing assay. Because this reporter had not yet been characterized in the liquid assay, we first visualized *irg-1p::GFP* expression in liquid without protective pre-treatment. When animals are fed the standard laboratory diet of OP50 prior to PA14 infection, we observed strong *irg-1p::GFP* expression in the pharynx in addition to the previously-described intestinal expression

induced by slow killing (Estes et al. 2010). Pharyngeal expression of *irg-1p::GFP* could be caused by mitochondrial damage in the pharyngeal muscle, as PA14 in liquid causes mitochondrial network disruption via iron sequestration by the siderophore pyoverdine (Kirienko, Ausubel, and Ruvkun 2015). As animals begin to succumb to infection-mediated damage, motility and pharyngeal pumping rates start to decline (M.-W. Tan, Mahajan-Miklos, and Ausubel 1999). Since significant death occurs far more rapidly during liquid killing than in slow killing, it is possible that PA14 cells build up in the pharynx as animals slow their pharyngeal pumping. Toxin-mediated damage to mitochondria present in the pharyngeal muscle could result in up-regulation of *irg-1* (Conery et al. 2014; Kirienko et al. 2013; Kirienko, Ausubel, and Ruvkun 2015).

Given the observation that neither *P. aeruginosa* nor *S. enterica* result in increased pathogen clearance, the increased survival caused by pre-exposure to these species is likely a result of decreased infection-mediated damage during liquid killing, as previously discussed in Section 4.1. A decrease in both pharyngeal and *irg-1p::GFP* expression in *P. aeruginosa* pre-treated animals may represent this. However, in *S. enterica* pre-treated animals, only pharyngeal expression is decreased, whereas intestinal expression is slightly increased in comparison to OP50 pre-treated animals. *irg-1p::GFP* expression is not induced by *S. enterica* grown on plates, so

this likely does not represent a compounding increase in expression due to pre-treatment, then exposure to PA14 (Estes et al. 2010). At this time, the significance of the spatial differences in *irg-1p::GFP* expression between the two pre-treatment conditions remains unclear. Although we have shown that RNAi-mediated knockdown of *irg-1* sensitizes animals to liquid killing, its exact function in the innate immune response is unknown.

Vitamin B12 improves mitochondrial network dynamics and increases survival in the liquid killing assay (Revtovich, Lee, and Kirienko 2019). As such, we were interested to see if addition of vitamin B12 to the pre-treatment media could down-regulate *irg-1p::GFP* expression during liquid killing. According to a previous report, animals reared on OP50 supplemented with exogenous vitamin B12 exhibit increased resistance to liquid killing (Revtovich, Lee, and Kirienko 2019). Here, we showed that OP50 grown on SKM supplemented with B12 was sufficient to reduce *irg-1p::GFP* expression during PA14 liquid killing, particularly in the pharynx. Although this does not prove that vitamin B12 is the sole factor responsible for resistance to liquid killing induced by pre-exposure to *P. aeruginosa* or *S. enterica*, it is consistent with previous work suggesting that *irg-1* is responsive to mitochondrial damage (Dunbar et al. 2012b; Y et al. 2014).

IRG-1 is predicted to contain a NADAR domain. This domain is also present

in *E. coli* YbiA, an enzyme responsible for the hydrolysis of the N-glycosidic bond in the first two intermediates of riboflavin biosynthesis, thereby preventing their toxic build-up (Frelin et al. 2015). Given *irg-1* is up-regulated upon response to specific cellular damage, it could be acting as a detoxification enzyme that prevents the accumulation of toxic by-products of metabolism (Dunbar et al. 2012a; Estes et al. 2010; Y et al. 2014). Since improving mitochondrial homeostasis through exogenous addition of vitamin B12 is protective against liquid killing, the aforementioned hypothesis could explain why addition of vitamin B12 to the pre-treatment media results *irg-1* down-regulation during infection, and why *irg-1* knockdown sensitizes animals to liquid killing (Revtovich, Lee, and Kirienko 2019).

Our results also suggest that a relatively small amount of vitamin B12 is sufficient to reduce *irg-1p::GFP* expression, given the lowest concentration of vitamin B12 (0.05 µg/ml) reduced fluorescence intensity to the same extent of plates supplemented with higher concentrations of B12. *C. elegans* imports vitamin B12 into cells using the ABC transporter *pmp-5*, so it is possible it has already reached saturation at 0.05 µg/ml, explaining why we do not see an further decrease in *irg-1p::GFP* expression at higher concentrations (Giese et al. 2020). Given that ZIP-2 regulated genes, including *irg-1*, are indispensable for survival in both slow and liquid killing,

and are responsive to agents that result in cellular damage, this branch of innate immunity may connect stress responses to the innate immune response (Dunbar et al. 2012b; Estes et al. 2010; Kirienko, Ausubel, and Ruvkun 2015; McEwan, Kirienko, and Ausubel 2012).

4.3 Vitamin B12 is partially responsible for *P. aeruginosa* and *S. enterica*-induced resistance to liquid killing

Worms reared on *E. coli* OP50 exhibit slight vitamin B12 deficiency, resulting in buildup of toxic TCA cycle intermediates in the mitochondria, namely, propionic acid. When starved of B12, methylmalonyl-CoA mutase (MMCM-1) cannot convert propionyl-CoA to succinyl-CoA, since ~~it uses~~ vitamin B12 is an essential cofactor. This results in disruption of the mitochondrial network and increased susceptibility to both infection and abiotic stressors. As such, vitamin B12-induced protection from liquid killing is dependent on MMCM-1 function (Revtovich, Lee, and Kirienko 2019). Using a reporter sensitive to propionate buildup, *acdh-1p::GFP*, we showed that *P. aeruginosa* is a far more abundant source of vitamin B12 than OP50. Reporter animals fed *S. enterica* exhibited *acdh-1p::GFP* expression that was nearly as high as that of OP50-fed animals, indicating *S. enterica* is also a relatively poor source of vitamin B12. To determine if increased vitamin B12 provision by each of these species underlies their ability to protect from PA14 liquid killing, we generated a *glp-4(bn2)*/*mmcm-*

1(ok1637) double mutant. When pre-exposed to *P. aeruginosa* and *S. enterica*, this strain still exhibited increased survival in the liquid killing assay, but less than animals with functional MMCM-1. Furthermore, mothers export dietary B12 to their offspring via the *mrp-5* transporter during embryonic development (Na et al. 2018). However, we showed that *P. aeruginosa* and *S. enterica*-mediated protection is not transmitted to progeny, as naive F_1 progeny of parents reared on either diet are not significantly protected from liquid killing compared to progeny reared on *P. aeruginosa* or *S. enterica.* From these data we can draw two main conclusions. First, the slight difference in vitamin B12 provision between OP50 and *S. enterica* pre-treatment is sufficient to cause MMCM-1-mediated protection from liquid killing. Second, enhanced provision of vitamin B12 is only partially responsible for the protective phenotypes observed, given an increase in survival was still evident in *mmcm-1* mutants.

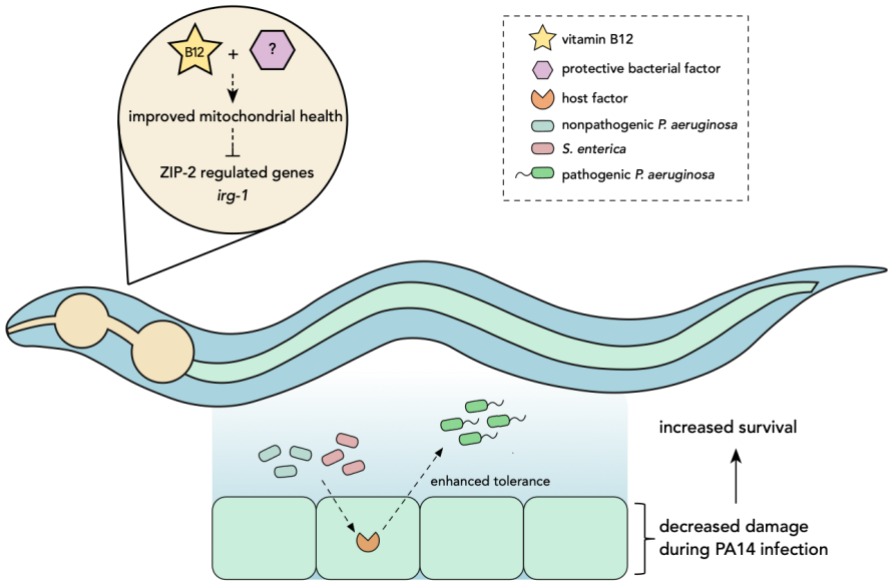

Figure 13. Proposed model of *P. aeruginosa* and *S. enterica*-induced protection from PA14 infection. Pre-treatment exposes *C. elegans* to vitamin B12 and a currently unknown, other protective bacterial factor(s) that reduces infection-mediated damage during liquid killing. Reduced damage during infection, either due to increased amounts of vitamin B12, or exposure to *P. aeruginosa* or *S. enterica*, results in lower expression of the ZIP-2 regulated gene *irg-1* in the pharynx.

CHAPTER 5. FUTURE DIRECTIONS

5.1 Uncovering host pathways mediating infection resistance

This work provides insight into ways in which exposure to different bacteria can modulate *C. elegans* susceptibility to pathogens. Many questions regarding the mechanisms underlying *P. aeruginosa* and *S. enterica*-mediated infection resistance still remain, namely, the bacterial and host

factors underlying this effect. On the host side, host defense response pathways could be explored. Since pathogen load does not change with pre-treatment, it is unlikely that up-regulation of antimicrobial effectors underlie protection. Future experiments could explore pathways responsible for clearing cellular damage, such as autophagy and mitophagy (Jia et al. 2009; Kirienko, Ausubel, and Ruvkun 2015; Jianhua Zhang 2013; Zou et al. 2014). Null alleles of key autophagic genes are embryonic lethal, and pre-treatment with *P. aeruginosa* or *S. enterica* precludes RNAi-mediated knockdown. Given these limitations, the role of autophagy could be investigated using fluorescent reporters that monitor the activation of *bec-1* and *lgg-1* (Kirienko, Ausubel, and Ruvkun 2015). Genes involved in mitophagy could also be investigated, such as *pink-1* and *pdr-1* (Ahier et al. 2021; Kirienko, Ausubel, and Ruvkun 2015). Null alleles and translational reporters of each of these genes are available from the Caenorhabditis Genetics Center (CGC). Crossing these into a *glp-4(bn2)* background to determine their role in *P. aeruginosa* and *S. enterica* mediated protection from infection. Measuring expression of selected stress-response or innate immune genes could also aid in understanding the transcriptional changes that occur during liquid killing upon protective pre-treatment. However, the disadvantage of using a targeted approach is it only allows the investigation of already-characterized pathways. To complement these analyses, an untargeted approach such as RNA-seq could be used to study global gene

expression changes during liquid killing between animals pre-exposed to *P. aeruginosa*, *S. enterica*, or *E. coli* OP50.

5.2 Identifying bacterial factors underlying *P. aeruginosa* and *S. enterica* mediation infection resistance

To identify bacterial factors underlying a particular *C. elegans* phenotype, transposon libraries of the strain of interest could be constructed and screened for mutants that result in a loss of phenotype (Diot, Garcia-Gonzalez, and Walhout 2018; Govindan et al. 2015; Watson et al. 2014). For this work, transposon libraries of a non-pathogenic *P. aeruginosa* strain, or *S. enterica* SL1344, could be used to identify mutants that do not protect from liquid killing. However, given the low-throughput nature of the method used to pre-treat then infect *C. elegans* in the liquid killing assay, it would not be feasible to screen thousands of transposon mutants for each strain. Here, we showed that supernatants of *P. aeruginosa* and *S. enterica* are not sufficient to protect from infection, whereas live lawns of these species are, indicating the bacterial factor inducing protection from infection is not secreted. Next, cell extracts could be generated of each strain and used to supplement OP50 lawns to determine if the lysis products can induce protection from liquid killing, or if live cells must be present. If the crude lysis products are able to induce protection, subsequent fractionation of each extract could be performed to identify bacterial factors underlying this effect.

In this work we show that PA14 load in the gut is not altered upon protective pre-treatment. However, this does not exclude the potential for PAO1 Δ*vfr* or *S. enterica* SL1344 to still be present in the gut during PA14 liquid killing. Co-colonization can be investigated by pre-exposing animals to fluorescently labelled PAO1 Δ*vfr* or SL1344. Using an mCherry-labeled strain of PA14, and PAO1 Δ*vfr* or SL1344 labelled with different fluorophore, such as GFP, co-localization of the two strains in the gut can be visualized (Rezzoagli, Granato, and Kümmerli 2019). Co-colonization could potentially alter the virulence phenotypes of PA14, either by precluding access to the intestinal cells by pre-emptive colonization during the pre-treatment period, or by microbe-microbe interactions that result in modulation of PA14 gene expression.

5.3 Understanding the regulation of *irg-1* in response to protective pre-treatment

The infection response gene *irg-1* is positively regulated by the bZIP transcription factor ZIP-2 in response to pathogenic *P. aeruginosa* (Dunbar et al. 2012b; Estes et al. 2010; Reddy et al. 2016). Upon toxin-mediated interruption of translation, or RNAi-mediated knockdown of tRNA synthetases or translation elongation and termination factors, *irg-1* expression is induced in a ZIP-2 dependent manner (Dunbar et al. 2012b). However, knockdown of translation initiation factors or histones results in

irg-1 induction independent of ZIP-2 (Dunbar et al. 2012b). *irg-1* is also expressed in response to agents that damage the mitochondria, however, whether or not this requires ZIP-2 remains unknown (Dunbar et al. 2012a; Y et al. 2014). Future experiments should focus on identifying the regulatory mechanisms underlying the decrease in *irg-1* expression during *P. aeruginosa* infection when pre-treated protective isolates or supplemented with vitamin B12. A ZIP-2::GFP translational reporter, as well as a *zip-2* null mutant, are both available on the CGC, so these can be easily obtained for future experiments. Given ZIP-2 is translocated to the nucleus upon activation, ZIP-2::GFP can be used to monitor the activation of ZIP-2 upon protective pre-treatment (Dunbar et al. 2012b). A null allele, *zip-2(ok3730),* can be introduced into *irg-1p::GFP* to determine if protective pre-treatment with PAO1Δvfr, *S. enterica*, or OP50 lawns supplemented with vitamin B12 is dependent on ZIP-2.

This study suggests that *irg-1p::GFP* can be used as a readout of pathogen-mediated damage. As such, this reporter can be used for future screens to identify factors that either reduce the virulence of *P. aeruginosa* or improve *C. elegans* ability to manage cellular damage accumulated during infection.

CHAPTER 6. REFERENCES

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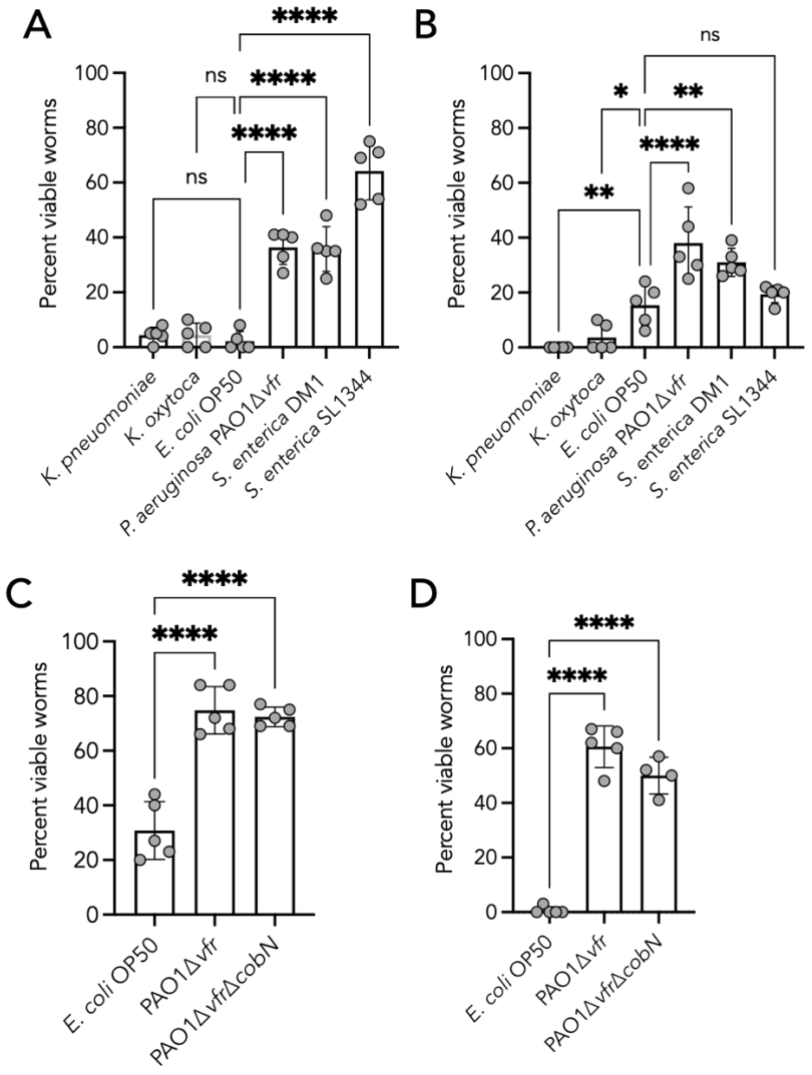
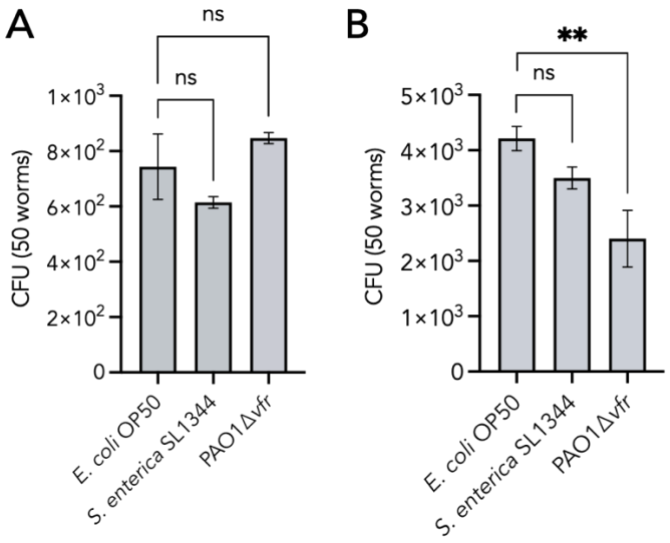
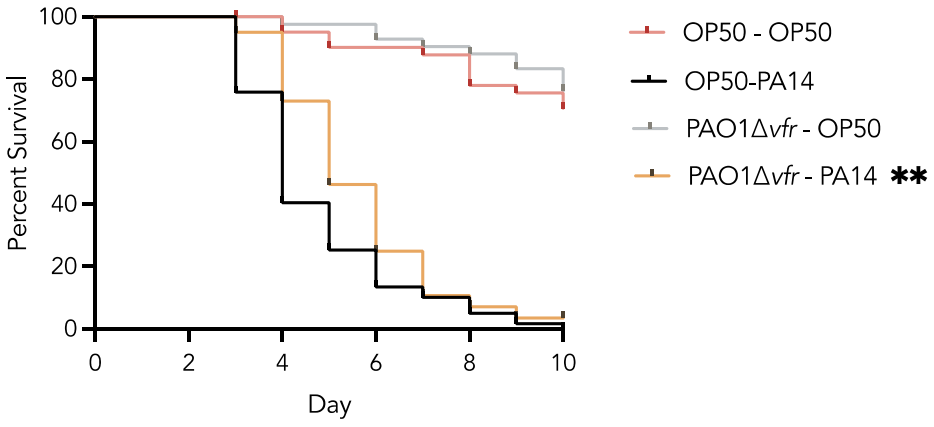
APPENDIX A. SUPPLEMENTARY FIGURES



Supplementary Figure 1. Liquid killing assay replicates. A – B. Pre-treatment with Gram-negative pathogens alters *C. elegans* susceptibility to PA14 liquid killing. **C – D.** Disruption of *P. aeruginosa* vitamin B12 biosynthesis does not impact survival in the liquid killing assay. Asterisks indicate isolates that significantly increased survival compared to OP50-pre-treated worms by One-Way ANOVA with Dunnett's multiple comparisons test. **** p<0.0001; *** p<0.001; ** p<0.01; * p<0.1.



Supplementary Figure 2. Colonization assay replicates. Pre-treatment with *P. aeruginosa* and *S. enterica* does not impact PA14 colonization during the liquid killing assay. Asterisks indicate isolates that significantly increased survival compared to OP50-pre-treated worms by One-Way ANOVA with Dunnett's multiple comparisons test. ** p<0.01.



Supplementary Figure 3. Pre-treatment slow killing assay replicate. Pre-treatment with PAO1Δ*vfr* prolongs survival in the slow killing assay. Asterisks indicate survival significantly different than OP50 - PA14 by Gehan-Breslow-Wilcoxon test. *** p<0.01.