The Evolution of Virulence in the Opportunistic Pathogen *Pseudomonas aeruginosa*

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Declaration

Unless otherwise acknowledged, the work presented in this thesis is my own. No part has been submitted for another degree at the University of Nottingham or any other institute of learning.

A. P. Day

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I would like to thank my family for their support during my study. I couldn't have got this far without them.

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Abstract

Understanding both the mechanistic basis of virulence and the evolutionary processes under which it can arise, is fundamental if we are to increase our knowledge of disease causing bacteria in an era of ever increasing antibiotic resistance. To date, there has been a substantial effort to understand virulence evolution both theoretically and experimentally. However, comparatively little experimental work has focussed on the evolution of virulence in opportunistic pathogens, and how virulence varies across multiple host organisms. In this thesis, the opportunist *Pseudomonas aeruginosa* was used to study virulence and its evolution in hosts. The virulence levels of different strains of *P. aeruginosa*, both laboratory and clinical, were tested in the nematode *Caenorhabditis elegans*. It was found that virulence of *P. aeruginosa* was lower in clinical strains isolated from chronic infections, and laboratory strains initially isolated from chronic infections, while a strain isolated from an acute infection was substantially more virulent. Using the *C. elegans* host, a selection experiment was carried out to test the effect of host density on virulence evolution. This resulted in a varied array of *P. aeruginosa* virulence phenotypes, but the worm evolved bacteria did not exhibit drastically different phenotypes from the worm negative control experiments. However, the degree of evolution observed across all treatment groups, compared with the wild type, highlighted the changes a bacterial population can undergo *in vitro*, even from a relatively short period of sub culture. Lastly, *P. aeruginosa* virulence was tested in alternative host organisms, *Galleria mellonella* (waxmoth larvae) and *Vigna radiate* (mung bean seedlings). Virulence varied according to the host, with

virulence in *G. mellonella* being universally high, while no virulence was observed in *V. radiate*. This work highlights that high or low virulence cannot automatically be assumed in different hosts for opportunistic bacteria such as *P. aeruginosa*. Furthermore, the results highlight the unpredictable nature of virulence evolution, and how dependent the emergence of virulence is on the interaction between the pathogen and the host. A more complete understanding of the principles underlying bacterial evolution and virulence could allow for the construction of more specific and accurate studies, with experimental conditions carefully engineered to best replicate the clinical conditions of interest.

Abbreviations:

3-oxo-C12-HSL	*N*-(3-oxododecanoyl)-L-homoserine lactone
AHL	*N*-acyl homoserine lactone
Amp	Ampicillin
C4-HSL	*N*-butanoyl-L-homoserine lactone
CF	Cystic Fibrosis
cm	Centimetre
CW	Chronic Wound
°C	Degrees Centigrade
FK	Fast Kill
g	Gram
GFP	Green Fluorescent Protein
h	Hour
l	Litre
LB	Lysogeny Broth
mm	Millimetre
mM	Millimolar
M	Molar
mg	Milligram
min	Minutes
ml	Millilitre
NGM	Nematode Growth Medium
nm	Nanometre
OD_{600}	Optical Density at 600 Nanometres
PQS	Pseudomonas Quinolone Signal

psi	Pounds Per Square Inch
QS	Quorum Sensing
rcf	Relative Centrifugal Force
Sm	Streptomycin
SNP	Single Nucleotide Polymorphism
SK	Slow Kill
SPE	Serial Passage Experiment
Tc	Tetracycline
µl	Microlitre
µm	Micrometre

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Chapter 1: General Introduction

What is a pathogen?

The organism which is the major focus of this thesis, *Pseudomonas aeruginosa* is habitually referred to as an opportunistic pathogen[1-5]. However, what does being an opportunist really mean, and how should this status be expected to influence virulence in host organisms?

Virulence has traditionally been viewed in a reductionist manner, in that the ultimate cause for virulence lies with the behaviour of either the host or the pathogen[6]. However, an alternate view is that virulence is a far more complicated emergent property, influenced by the unique biology of each host and microbe, as well as how they interact on a case by case basis[7-10]. Under this hypothesis, because disease occurs as a result of unique host-microbe interactions, virulence is inherently novel, unpredictable and cannot be reduced to first principles[7]. The influence of the host on the disease process can extend far beyond the expected 'how does the pathogen avoid being cleared by the immune system' approach. For certain infections, the host response itself is the primary driver of disease symptoms, rather than an active process by the microbe. Examples of this include S*treptococcus pneumoniae* and *Mycobacterium tuberculosis*, where the bacteria in the lungs does not directly inflict harm, the inflammatory response the infection induces causes the damage, rather than a microbial virulence factor[9, 11]. This is therefore a ready explanation for the virulence of certain microbes, such as *Staphylococcus epidermidis* and *Candida albicans*, which are commonly non-pathogenic but can

induce severe disease under certain circumstances[8]. Even the classical molecular biology focus on virulence factors may not be entirely reliable. While it is not in doubt that certain factors do enable some microbes to invade and colonise hosts, this does not cover all eventualities. Some microbes, such as *Aspergillus fumigatus,* appear to lack major virulence factors, yet are still capable of instigating disease[9].

This challenge of linking virulence factors to microbial pathogenicity is particularly important in the light of the recent push towards targeting virulence factors themselves as an antimicrobial therapy[12]. Such therapies could be useful in cases where the disease is actually the result of virulence factors, such as has been established with *Vibrio cholerae* and cholera toxin[13]. However, even when dealing with established virulence factors, this 'anti virulence' approach may not result in successful treatments. For example, the *lasRI* quorum sensing system (QS) in *P. aeruginosa* has long been described as a major regulator of virulence[14]. So, inhibiting an element of the *lasRI* signal pathway would seem like a viable way to combat an infection. However, *lasR* mutants are known to occur naturally in the cystic fibrosis lung environment[15]. Therefore, targeting QS signalling in such an infection may be futile, as the bacteria already lacks the required receptor to respond to the signal, yet remains pathogenic in cystic fibrosis patients. Although a recent study has reported that QS can remain active, even in some LasR-null clinical strains, which suggests that broad-range inhibition, targeting the full array of QS signalling systems might have better success than targeting a specific 'master' virulence regulator, such as *lasR*[16].

Additionally, targeting virulence factors in a microbe where disease symptoms are due to the host immune response, such as M*ycobacterium tuberculosis*[9], could be a wasted effort, as the therapy would not be targeting the true source of virulence. However, this should not detract from the circumstances where such treatments could be effective, and they certainly are an avenue with promise[12].

Instead of classifying microbes as simply pathogenic or non-pathogenic, an alternative system has been proposed, in which microbes are assigned to one of six classes (Table 1.1), depending on whether disease is a result of host immune response, intrinsic pathogen characteristics, or a combination of the two[11]. Under this system, *P. aeruginosa* is class 2, capable of causing damage by both methods. [11].

Class	Nature of Damage	Example Organisms
1	Low intrinsic virulence, only cause damage in hosts with a weak immune response.	*Legionella pneumophila* *Staphylococcus epidermidis*
2	Cause damage by both host- and pathogen-mediated mechanisms. Can damage normal hosts but severe infections are associated with impaired immune function.	*Clostridium tetani* *Pseudomonas aeruginosa* *Vibrio cholera*
3	Cause damage by both host- and pathogen-mediated mechanisms. Can cause severe damage under both weak and strong immune responses.	*Streptococcus pyogenes* *Mycobacterium tuberculosis* *Staphylococcus aureus*
4	Cause damage only at the extremes of immune response, both weak and strong. Normal immune response does not damage host.	*Aspergillus* spp. Vaccina virus
5	Cause infections resulting in pathogen-mediated damage, but damage can be enhanced by excessive or inappropriate immune response.	*Mycoplasma pneumoniae* *Campylobacter jejuni* Poliovirus
6	Only cause damage in conditions of strong immune response.	*Helicobacter pylori*

Table 1.1: The 6 classifications of disease causing microbes[11].

Under this revised way of examining disease, there is no such thing as an 'opportunistic' pathogen, just as any given microbe cannot be definitively classified as pathogenic or non-pathogenic, thanks to the variable and emergent nature of pathogenicity[8, 11]. Attempts to classify microbes in the traditional manner, including as opportunists, are attributing properties solely to the microbe, when pathogenicity is truly due to the combination of the 3 factors of individual host, microbe and their interactions[8]. So the question when dealing with pathogenicity and virulence becomes 'Does interaction between hosts and parasite damage the host, and if so how?', rather than 'What does this microbe do to the host?'[9]. When pathogenicity is considered from this combined viewpoint one of four outcomes occurs: commensalism, colonisation, latency or disease[8]. Of course, a change in individual circumstance could cause host-microbe interaction to change, thus allowing an ordinarily commensal microbe to instigate disease. Therefore, the traditional classification of *P. aeruginosa* as an opportunistic pathogen becomes redundant under this model, as there is no inherent difference between a so-called opportunist and any other microbe. Disease will only occur if the required set of host-microbe interactions take place, and this will depend on individual traits of both, rather than species-level microbial attributes[8].

Explaining virulence, the historic view

In the early years of the 20th century it was proposed that pathogens evolve towards benign virulence over time[17]. This 'avirulence hypothesis' dominated ideas about the evolution of virulence for most of the last century, eventually

reaching the point where it was considered 'conventional wisdom'[18, 19]. The hypothesis explains the existence of highly virulent, deadly pathogens as being recent interactions with hosts and are therefore young pathogens that have not had to time to achieve benign cooperation or commensalism with their hosts. However, to a modern viewpoint this hypothesis seems optimistic and naive. The error in assuming that pathogen evolution would automatically be beneficial to the host was even alluded to in *On The Origin of Species* itself, "What natural selection cannot do, is to modify the structure of one species, without giving it any advantage, for the good of another species"[20]. So, whilst a benign pathogen may be of more benefit to a host, the same is not necessarily true for the pathogen. Indeed, despite widespread acceptance for most of the 20th century, the avirulence hypothesis began to be challenged relatively soon after it was first proposed[21, 22].

High virulence can and does occur in the manner initially suggested by the avirulence hypothesis. New pathogens can emerge by jumping species, with three quarters of emergent pathogens having zoonotic origins[23]. Pathogens which jump from different host organisms can be extremely virulent thanks to their evolution in a different host species[24], and a lack of host adaptation to the new threat[7]. A recent example of this process is the Ebola virus, which is believed to jump to humans from its natural reservoir in fruit bats[25]. However, we cannot automatically assume that Ebola will inevitably evolve towards attenuated virulence and benign cooperation with continued exposure to humans.

The trade-off hypothesis

The central idea behind the avirulence hypothesis (visualised in Figure 1.1) is that killing the host is not beneficial for the pathogen, as killing a host will also kill any remaining pathogen population in that host. However, this hypothesis does not fully consider how evolution works. What drives evolution is not the survival of individuals, it is about maximising fitness and therefore successfully propagating genes into the next generation. Allelic variations that improve reproductive success will therefore be evolutionarily favoured. If high virulence allows a pathogen to infect a greater number of hosts, then the degradation and death of the original host organism doesn't matter. In this scenario, a pathogen's genes are passed on to a greater number of offspring because of successful transmission to more hosts, resulting in more copies of genes involved in virulence being present in future generations[18]. This principal has been outlined mathematically[24] and experimentally[26].

As an alternative to the avirulence model, a trade-off hypothesis was proposed. Increased transmission results in higher parasite fitness. Therefore, in isolation, ever higher transmission rates would be evolutionarily favoured. However, increases in transmission are often a result of increased virulence, meaning that transmission and virulence are evolutionarily linked. This can decrease the fitness of the pathogen, as it damages the host, causing a degradation of the host's ability to support the pathogen and reducing opportunities for transmission to fresh hosts[18, 19]. Therefore, there is an evolutionarily equilibrium point, unique to every pathogen and host interaction, where host exploitation and

duration of infection are balanced to maximise the reproductive potential and fitness of the pathogen.

Examining the evolution of pathogen virulence is more complicated than simply looking at the evolution of the pathogen because of the host-pathogen dynamic. Both organisms exert selective pressures on each other. This coevolution can influence a wide variety of traits in both host and pathogen, including population dynamics, diversity, sexual reproduction and parasite virulence[27]. At first glance, the evolutionary dynamic for the host is simple, a host that develops greater resistance to the pathogen will be at a competitive advantage against non-resistant organisms. However, this is not always the case. Pathogen resistance may carry fitness costs, such as lower growth rates for resistant hosts[28], which would give non-resistant organisms the competitive advantage in the absence of the pathogen. The opposite can also occur, where the presence of a pathogen permits coexistence between different variants of the host. If one variant would normally be the superior competitor, but is more susceptible to infection by the pathogen than the less competitive variant, the pathogen's presence can allow both variants of host to persist, rather than the stronger competitor displacing the weaker[28]. Over time this evolutionary arms race between host and pathogen can have both advantages and disadvantages for the host. While an evolved resistance can also protect against infection by similar related pathogen groups, this can result in lowered resistance if it ever interacts with the ancestral pathogen again, as the host has become over-specialised[29]. This specialisation of pathogen to host decreases in spatially heterogenous

environments, a trait which has been attributed to the increased costs of generalism when exposed to varied environmental conditions[30]. In other words, increased specialisation to one area of a varied environment would disadvantage the pathogen in others, so generalism occurs as the least costly evolutionary outcome overall.

Pathogens also have a set of evolutionary trade-offs, two of which are key underpinnings of the trade-off hypothesis and the optimisation of pathogen transmission. These are (1) transmission period (most commonly the lifespan of the infected host, or the time taken for a host to clear the infection) and (2) transmission rate (including the spread of a pathogen to new hosts and the rate of pathogen reproduction). Increased transmission rate is often a function of increased virulence[31, 32], which damages the host, decreasing the transmission period[33]. Two mechanisms have been proposed to explain this: (1) selection favours fast transmission when a pathogen is virulent, or (2) the increased virulence evolves due to increased levels of horizontal transmission (between host) relative to vertical (in host)[24]. Virulence has also been linked with recovery time for non-lethal infections, with low virulence associated with quick recovery timers and lower transmission rates[34]. The interplay between these factors results in an optimal level of virulence, where speed of transmission and host degradation are balanced[35, 36]. However, this balance is not implicitly benign. If a high level of virulence allows for a transmission rate fast enough to compensate for the limited transmission period, or if host degradation does not deleteriously affect parasite transmission, severe disease

can be evolutionarily favourable for the pathogen[18, 32]. Examples of pathogens that follow the high transmission, high mortality include feline calicivirus, myxoma virus, H5N2 influenza in avian species and classical strain *Vibrio cholerae*[37-40]. Pathogens taking the alternate pathway of low mortality and a large infection window include human rhinoviruses and El Tor strain *V. cholerae*[40, 41]. Both pathways are visualised in Figure 1.1. But perhaps the most extreme end of this equilibrium could be the nitrogen fixing bacteria found in the root nodules of certain legumes. While ostensibly a textbook example of mutualism, both organisms are still mainly concerned with their own survival, and certain legumes have been shown to maintain the ability to divest themselves of the symbiotic bacteria in high nitrogen environments, an ability which potentially helps forestall the emergence of parasitism[42]. So even at the far end of the spectrum, host-microbe interactions should be viewed, at best, as a marriage of convenience from the host's perspective.

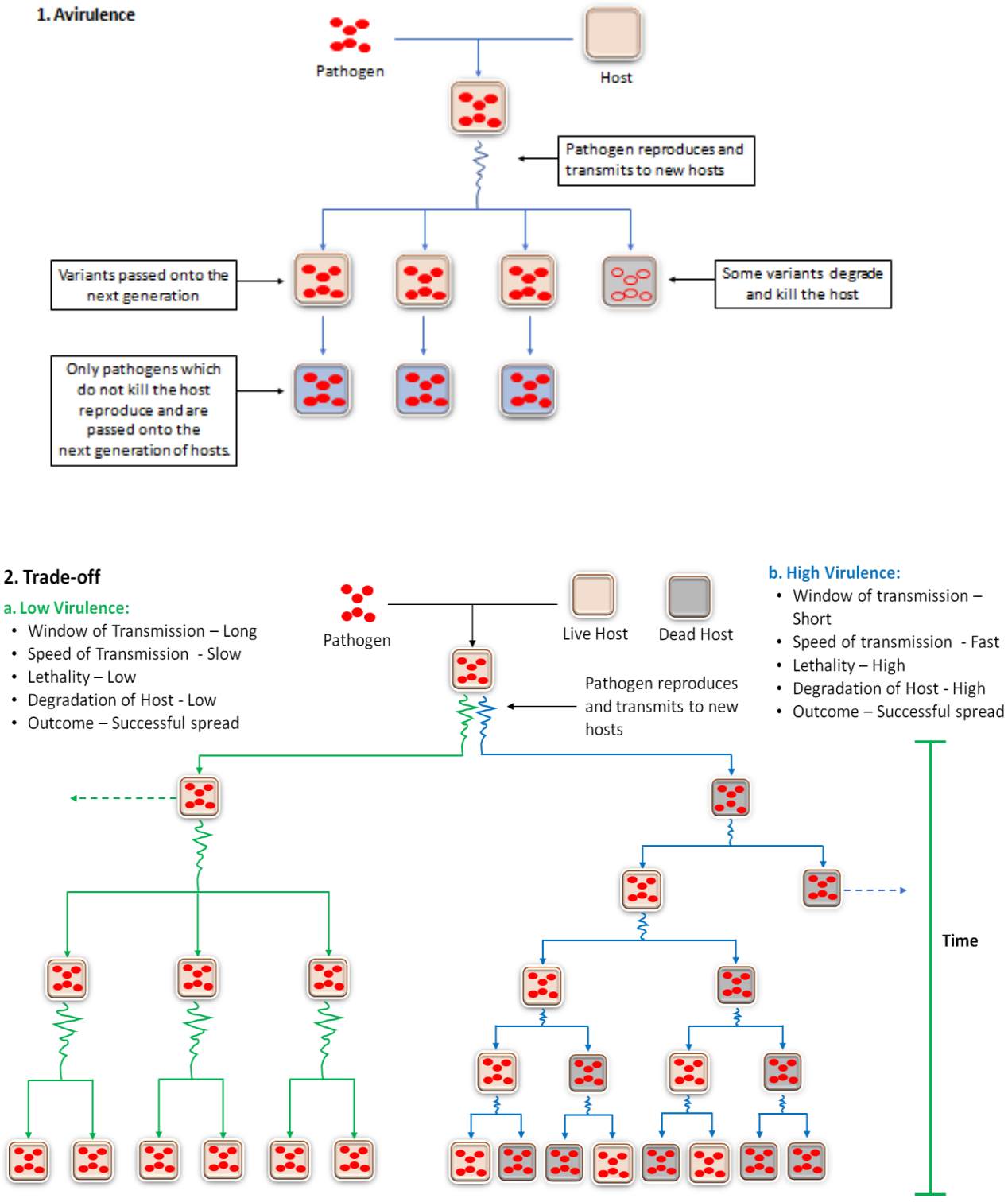


Figure 1.1: Comparison of different transmission hypotheses. In the avirulence model (1.), host degradation is harmful to the long-term success of the pathogen by preventing transmission to fresh hosts. In the trade-off model (2.), both high and low virulence can be evolutionarily favourable, as faster host death is not inevitably a dead end, but is balanced by a more rapid transmission to fresh hosts.

Additions to the trade-off concept of virulence evolution

While fitting into the overall framework of the trade-off hypothesis, a further level of pathogen evolution was proposed, with pathogens competing both in transmission to fresh hosts and between each other in established hosts[24].

The first of these, competition in transmission, adheres very closely to the central hypothesis of this study. That is, that low host density environments will favour the evolution of low virulence phenotypes, while high host densities will favour higher virulence and virulence retention. For some pathogens the direct benefit of virulence is clear, the symptoms of infection aid the spread of infection, such as the diarrhoea caused by cholera, or coughing/sneezing in the case of respiratory pathogens[18, 24]. However, some pathogens have 'neutral' virulence, where virulence levels are irrelevant to the transmission of the pathogen. This could occur due to the pathogen being in a different host to the intended one. Alternatively, virulence could manifest after the selectively relevant period of transmission, such as in the case of chronic Hepatitis B, as liver cancer only ensues decades after infection. In this case the pathogen has most likely had its best opportunity to infect fresh hosts long before severe adverse symptoms emerge[24].

Regarding the idea of virulence evolution being driven by intra-host competition, 4 possible models have been proposed[24]. Firstly, virulence may evolve via diversity thresholds. Under this model virulence is a collective property tied to the extent of pathogen polymorphisms present in the host. The large number of variant pathogen genotypes create an environment of high competition which

favours rapid pathogen growth. Virulence occurs when the host's immune system becomes overwhelmed by the many types of pathogen it is forced to address. This model arose to explain the development of AIDS from HIV infection[43].

The second option, dubbed the associated model, is generally considered to be the most common outcome. It is based on the premises, that some pathogen genotypes are better adapted to in-host growth, and that faster growth generates higher virulence[24]. This is the model that is most consistent with the trade off hypothesis, providing the underlying explanation behind the 'high transmission, high virulence, low infection window' side of the spectrum. However, the remaining two proposed models stand as potential alternatives to the associated model.

The reversed replication and virulence model is based around the observation that pathogen growth rate does not always increase with increased virulence. In fact, the opposite was reported, with faster replicating pathogens displaying lower virulence[44]. However, this phenomenon has only appeared in a particular type of virus, defective in many viral genes, which have a competitive advantage when in the same cell as normal viruses. This model would appear to represent a form of evolutionary 'cheating', as the full virus is also required for a competitive advantage[24], suggesting that there is something necessary to full function that the faster replicating and damaged virus lacks. Therefore, this model isn't really applicable to the overall trade-off hypothesis, as it only applies

to a very specific type of pathogen and relies on within-host interactions that appear to be independent of transmission to fresh hosts.

Some pathogens, in particular viruses, exhibit tissue tropism, where they are only virulent in certain tissues of the host organism[45, 46]. These pathogens form additional, more virulent niches by colonising more types of host tissue, or they can shift towards longer transmission windows by reducing rate of replication in the 'target' tissue, while retaining high, potentially infectious populations in other tissue types[24]. Just like the reversed replication model, this model is rather specific, but does not contradict the trade-off hypothesis, as it does not address the central concept of exchanging virulence for longer in host. The type of within-host competition outlined here could produce a pathogen anywhere on the trade-off equilibrium, depending on the nature of the specific pathogen in question.

There is also an alternate evolutionary path that could superficially resemble the avirulence hypothesis. If a host population is capable of supporting a substantial pathogen population in addition to a sustainable population itself, this could look like the host-pathogen system is progressing towards symbiosis. However, when this effect has been reported in bacteria-phage interactions, the effect is actually attributed to phenotypic resistance[47], and so is a function of increased resistance on the part of the host and not achieving symbiosis with the pathogen.

Although benign interactions between host and pathogen are not inevitable, neither are they impossible. It has been proposed that the extent to which

benign interactions will occur depends on the degree of overlap between host and pathogen reproductive success. So, a pathogen that spreads through vertical transmission is more likely to develop commensal behaviour, as it shares its reproductive future with that of its host[48]. Alternative explanations for increased attenuation of a pathogen in a host have also been put forward. Evolution in a host many increase the pathogen's ability to evade the host immune response[49], reducing virulence, especially when that virulence is inherently tied to an immune response, such as severe inflammation[11]. It is also possible that lowered virulence may be the result of the pathogen fluctuating between within-host and outside-host environments, the challenge of surviving in multiple environments providing a limit on how specialised and virulent a pathogen can become. This could be expected to be an especially common situation for so called opportunistic pathogens, as these are often capable of surviving in the absence of host organisms[49].

Alternative Explanations to the Trade-off Hypothesis

There are challenges to aspects of the trade-off hypothesis, although they tend to be adding layers of complexity to the model, rather than flat out refuting it. It has been reported that a different set of evolutionary pressures may be involved in durable infections. In these infections a reduction in transmissibility is only slightly deleterious, whereas reducing host lifespan would be far worse for the pathogen[50]. However, this appears to assume that the loss of individual hosts is always bad for the long term survival of the pathogen, which is not necessarily the case[32, 51]. This conclusion could simply be the observation of pathogens

already on one end of the equilibrium, specialised towards long infection windows rather than high transmissibility.

While a form of this trade-off equilibrium is most likely present for all host-pathogen systems, there is no inherent uniformity regarding how individual pathogens will act. It is all determined by the combination of individual environmental and genetic factors of the pathogen in question (the genetics of the host are essentially additional environmental factors when considering pathogen evolution). For example, a pathogen with more robust genetic systems (such as better repair machinery or more stable homeostatic feedback loops) reduces the pathogen's sensitivity to environmental factors[52, 53], as it is harder for the pressure from the environment to influence a 'tougher' system. This reduction in selective pressure can result in increased variability in the pathogen biology, as there is more leeway for inefficiency in any given component system[53].

It is predicted that a system with protections against variability in its underlying components will possess a higher level of stochastic variability in those components[54]. For example, if a bacterial population is secreting high levels of a given exogenous molecule, then it could actually support a variety of different production levels, as there's enough to go around, even if some of the population aren't contributing equally. This is the niche that so called 'cheats' exploit[55]. However, this changes if the system in question works at a cellular, rather than population level, for example membrane bound uptake receptors. Observed stochasticity in these systems should be relatively low, as each

individual requires their own version and 'cheating' offers no benefit[54]. Overall it is predicted that evolutionary dynamics will drive systems towards multiple rounds of enhanced system robustness and component decay[53]. However, the ultimate result of this is that the development of less efficient components can be a competitive advantage, as they reduce overall costs and could result in a more efficient overall population, and will therefore be evolutionarily favoured[54].

However, this advantage may only be a short-term gain, with potentially harsh consequence for the organism in the long term. While initially offering a competitive advantage thanks to lower metabolic load, this protective effect of robust control networks can allow for longer term accumulation of deleterious mutations[52], leading to an eventual degradation of pathogen fitness, especially if environmental conditions become harsher. Under these conditions, it might be expected to see a massive drop in fitness, rather than a gradual decline, as the increasingly harsh environment finally overcomes the buffering effect of robustness, and the accumulated mutations all 'kick in' at once.

The severity of host resistance, defined as the capacity of a host's genotype to control pathogen replication[56], is a major facet of the environment that pathogens, and their associated virulence, must evolve in. Artificial boosting of host resistance, such as with vaccination and drug treatment, has been shown to favour more virulent pathogens during co-infection[57-59], as this upgrading of the arms-race between host and pathogen will leave any surviving pathogen more dangerous, especially if it subsequently interacts with hosts lacking the

artificial resistance boost. However, there is also evidence for the opposite, that exposure to less resistant hosts can foster highly virulent pathogen capable of infecting hosts fully resistant to the ancestral pathogen[60]. High levels of host resistance are not always beneficial for the host. The more extreme immune response can carry a fitness cost itself, as it increases the degree of immunopathology the host experiences[56]. One would expect the degree this occurs to depend somewhat on the nature of the increased host resistance, as something like a vaccine is not supposed to induce a heavy immune response. It has also been shown that pathogens with increased virulence under conditions of higher host resistance only display that heightened virulence when in the host genotype it is adapted to. Mean virulence fell across the totality of host genotypes, presumably due to over specialisation by the pathogen towards one single genotype[56]. As is so often the case with the evolution of virulence, there is not one easy explanation for all eventualities, instead it appears the effect of high host resistance on virulence evolution will depend on the specific nature of the host and pathogen in question.

Cooperation Between Individuals in a Pathogen Population

Another contributing factor to the evolution of pathogenic organisms is the cooperation between individuals in a population (behaviour that benefits other individuals, and is maintained, at least in part, because of that benefit). It has been argued by some that cooperation should be evolutionarily favoured, as that is the way to maximise benefit at the population/species level[61]. However, just like the avirulence hypothesis, this hypothesis does not stand up to the realities

of evolution. A specific individual often finds itself in both intra-species and inter-species competition, so if an allele provides a competitive advantage over other individuals of the same species, one would expect those alleles to predominate. This is especially true regarding cooperative, social behaviours, as individuals (sometimes referred to as 'cheaters'[62]) can benefit from social behaviours without contributing[63-65]. Cheating should undermine cooperative behaviours, and yet this is clearly not the case in nature because cooperation in microbes is common[66, 67]. One way for cooperation to be evolutionarily stable is via kin selection, because as long as relatives benefit from the social behaviour, the genes responsible are passed on, even if the altruistic individual is disadvantaged[64]. This has been found to be the case for bacterial virulence, where higher relatedness in a population facilitates more cooperative exploitation of the host, resulting in higher virulence[68].

Alternatively, cooperation can evolve if there is a way for the benefits of cooperation to be directed back towards the co-operator (or potentially its relatives)[69]. It is also possible for cooperation to arise, even between 'cheating' populations, if the public goods are linked. Under this situation, lack of one public good may increase production of another, resulting in increased specialisation of public good production and a stabilisation of cooperation[70]. These forms of cooperation are illustrated in Figure 1.2. There may also be inherent limits of the extent that microbes can cooperate. Cooperation has been reported as increasing the instability of microbial communities, as it can lead to runaway positive feedback loops that disrupt the community's equilibrium[71].

Also, some level of cheaters within a population may actually be advantageous to long term survival, with periodic population collapse due to cheaters as part of cycle which preserves cooperation in the long term[67]. So, just like other bacterial behaviours, evolution of extreme cooperation phenotypes may be naturally selected against.

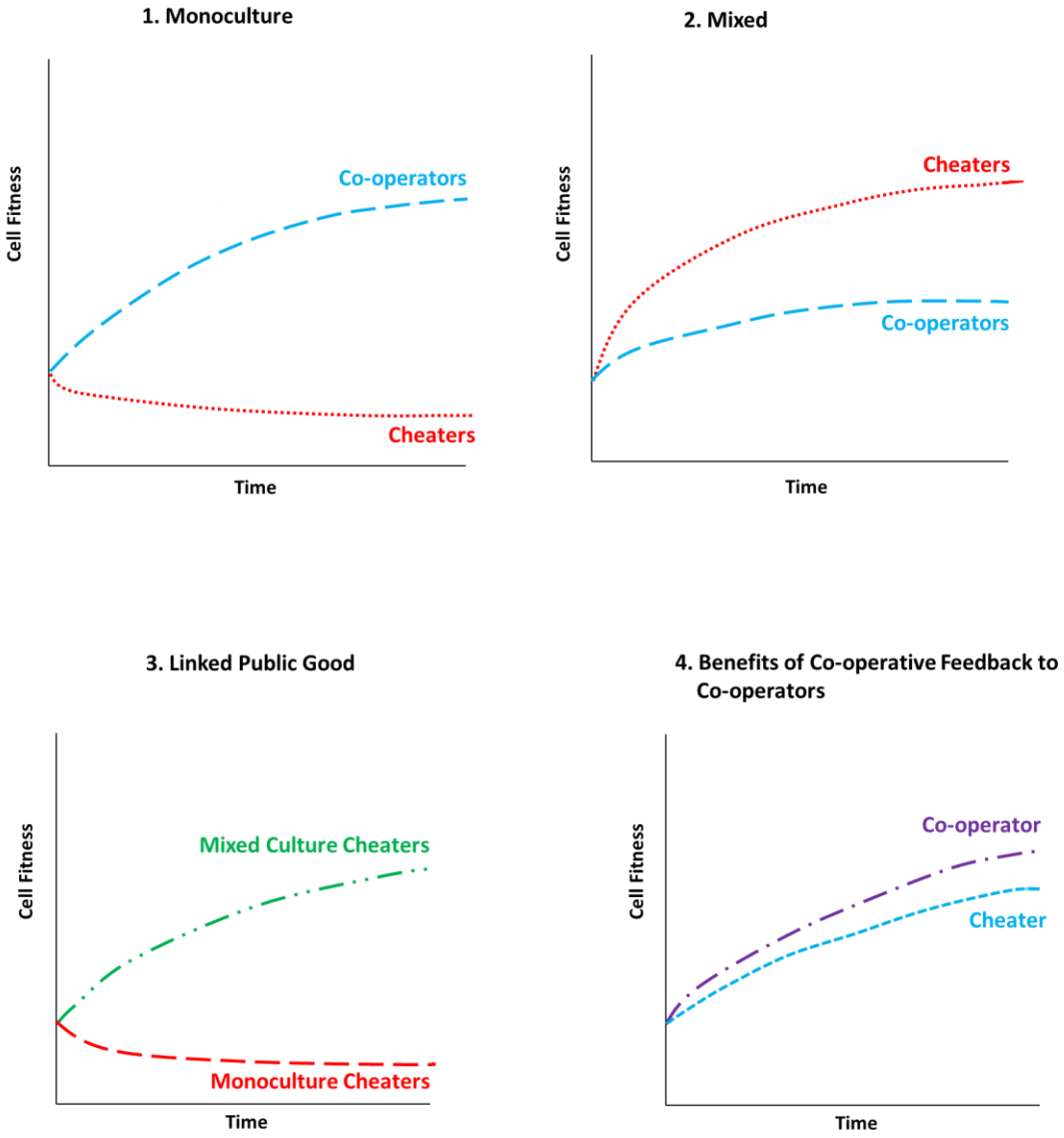


Figure 1.2: Illustration of cooperation models. In the most common view, cheaters are at a disadvantage in monoculture (1.), but perform better than co-operators in mixed culture (2.). When production of different public goods are linked, different strains of cheaters can produce cooperating populations (3). Lastly, if cooperating strains (or their descendants) can also directly benefit from the cooperation, then co-operators will not be outcompeted by cheating strains (4.). Graphs 1. and 2. are adapted from a previously published figure[72]. 3. and 4. are new graphs derived from information from Ross-Gilespie et al.[70] and West et al.[69] respectively.

Serial Passage Experiments and the study of Pathogen Evolution

Serial passage experiments (SPEs) have been used as a tool to study the evolution and adaption of pathogens for many years, and the general consensus has been that serial passage in host increases virulence[33]. However, many of the studies used to inform this general conclusion involved artificial transfer, where the parasite was deliberately moved to fresh hosts by the researchers. This is a potentially flawed methodology for the study of virulence evolution, as it removes one side of the trade-off balance. If transmission is always assured thanks to human intervention, there is no selective disadvantage for virulence shifting towards increased lethality. With nothing to impede their progress, it is unsurprising that more virulent, faster growing strains of pathogen come to prominence in such studies. The same process was observed during the 20th Century, when a mild form of malaria parasite was given to patients to induce fever during treatment for syphilis. Yet the malaria quickly evolved increased virulence to the point it became more harmful to the treated individuals than the initial syphilis infection[18]. It has also been suggested that transitioning to, and evolving in, a new host would attenuate virulence in the older host[73-76]. Under this hypothesis host specialisation, rather than generalism would be the normal evolutionary path for a pathogen to take. This hypothesis is supported by studies which found serial exposure to one host genotype caused fitness trade-offs which left the adapted pathogen less suited to a new, unfamiliar, host genotype than unadapted pathogen[77, 78].

Opportunistic Pathogens and Evolution

Despite the recent challenges to how we view pathogenicity[7-9, 11], the idea of opportunistic pathogens has been present for a long time. Such pathogens are classically characterised as organisms that can become pathogenic following a perturbation to their host. Alternatively the term is used to refer to pathogens which are both disease causing in humans and zoonotic, exploiting a variety of hosts[79]. They present a particular set of challenges when discussing virulence evolution, as these pathogens commonly spend time in the wider environment, resulting in non-parasitic contexts which must be balanced with adaption to the in-host environment[79]. The extent to which any given opportunist will have to balance these factors is a unique function of their likelihood to transfer between environments, so an opportunist that frequently transfers between hosts (or host and wider environment) is more likely to adopt a generalist phenotype, while one which rarely encounters varied environments has a greater opportunity to become more specialised[79]. One of the most studied opportunistic pathogens is *Pseudomonas aeruginosa*.

Pseudomonas aeruginosa

P. aeruginosa is a Gram-negative bacteria, ubiquitous in the environment. It is an opportunistic human pathogen[80] and is a leading cause of death among surgically injured or immunocompromised hospital patients[81], as well as Cystic Fibrosis patients[82]. *P. aeruginosa* has the ability to express a wide range of virulence factors during an infection, many of which are under the control of quorum sensing[83-85].

Quorum sensing (QS), density dependent cell-cell communication, is a major factor in regulating *P. aeruginosa* virulence[84-87]. While multiple QS systems are present in *P. aeruginosa*, two major QS pathways are its pair of LuxRI homologs, *lasR/lasI* and *rhlR/rhlI*. These two systems regulate the production and response to *N*-acyl homoserine lactone (AHL) signal molecules[88, 89]. Each of the LuxRI homologs produces and utilises a different AHL. *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) is the AHL associated with the *lasR/I* pathway, while *N*-butanoyl-L-homoserine lactone (C4-HSL) is associated with *rhlR/I*[90, 91]. Of these two QS pathways, the *las* system is thought to be the dominant system[16, 88, 89]. This is due to the regulatory role that the *lasRI* gene products have on the regulation of both the *rhlR* gene and the receptor it encodes[88, 89, 92]. A third QS system in *P. aeruginosa* is regulated by 2-heptyl-3-hydroxy-4(1H)-quinolone (The Pseudomonas quinolone signal; PQS), which regulates some *rhl*-dependent virulence determinants[4]. PQS has also been implicated in the regulation of apoptosis, as mutants with pronounced lysis phenotypes were found to overexpress PQS[93].

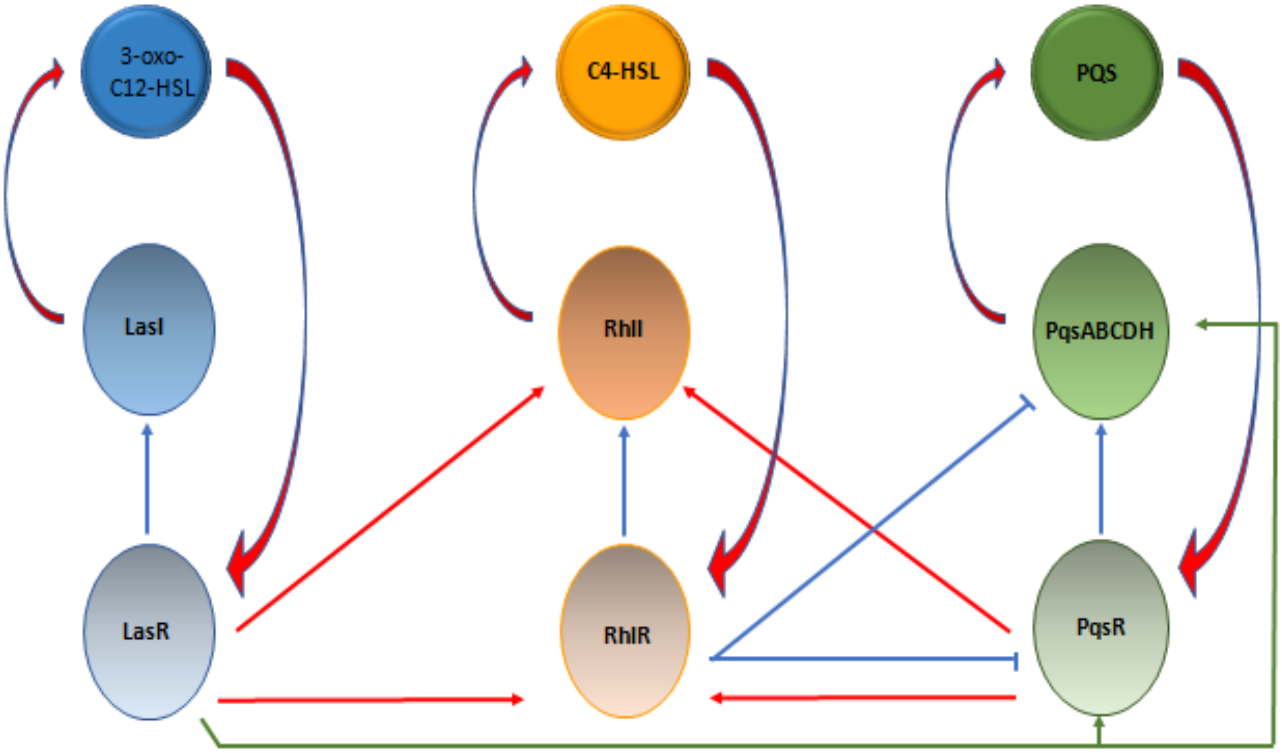


Figure 1.3: Visualisation of the QS hierarchy of *P. aeruginosa*, adapted from Papenfort et al.[89]. LasR controls RhlR and PqsR, and hence is viewed as the key QS regulatory gene.

LasR, and its role in the virulence of *P. aeruginosa* in the CF lung has been heavily studied, with *lasR* mutations commonly found in the lungs of patients[94, 95]. However, this supremacy of *lasR* as the key *P. aeruginosa* QS gene, particularly in the CF lung has recently been challenged. It has been reported that mutations in *lasR* do not necessarily result in the expected changes in RhlR activity[16]. This suggests that the *P. aeruginosa* system may be more adaptable and robust than had previously been thought; the established QS regulatory pathways can be uncoupled to retain some level of QS activity, even if regulators higher up the pathway are lost. The same study also found that clinical *lasR* mutants are not always LasR-null, meaning that LasR activity can be retained, even in clinical strains that we would have previously assumed would be LasR negative. It is suggested that this adaption allows *P. aeruginosa* to get the best of both worlds, able to access LasR-controlled gene products by alternate means when necessary, but the lack of the usual pathway results in a lower base metabolic load, and hence a competitive advantage[16].

Pyoverdins are high affinity fluorescent peptidic siderophores. Although three types of pyoverdin are known, only types I and II are found in *P. aeruginosa*, and only a single type is produced by any given strain[96]. Pyoverdin functions as the principal siderophore in *P. aeruginosa* and is an important virulence factor, being crucial for virulence in certain models, such as burn wounds[97] and a significant factor in virulence determination in others, such as *Galleria mellonella*[98]. It has also been demonstrated to be a key virulence factor in the killing of *Caenorhabditis elegans*[81, 99]. There is also a second siderophore in *P.*

aeruginosa, pyochelin. While pyoverdin is the siderophore of choice when iron is severely limited, *P. aeruginosa* switches to pyochelin production when iron is more plentiful. This process is achieved by pyoverdin repressing pyochelin production, and a negative feedback pathway represses pyoverdin in higher iron environments[100]. However, pyoverdin remains the primary siderophore linked with virulence, while pyochelin has been reported as being less important[101]. Siderophores have also been found to promote the build-up of pro-inflammatory cytokines and chemokines in *Klebsiella pneumoniae* infections, and it is speculated that the same effect may occur with other siderophore producing pathogens, although whether this applies to *P. aeruginosa* is currently unknown[102].

P. aeruginosa also produces hydrogen cyanide as another virulence factor in its arsenal[103-105]. Biosynthesis is controlled by 2 independent systems, the Rhl QS system, and elements of the alginate production pathway[105]. The activity of cyanide has been implicated as an important virulence factor in multiple host organisms, including both humans and *C. elegans*[104, 105].

The phenazine pyocyanin is another major virulence factor in *P. aeruginosa*. It is coded by 2 separate operons, which are 98% identical, but under the control of different promoters[106]. The production of pyocyanin is necessary for *P. aeruginosa* to achieve full virulence[107]. It has also been linked with persistence of *P. aeruginosa* infections, as well as the induction of neutrophil apoptosis[1]. It is also implicated in the killing of *C. elegans*, though the absence of pyocyanin

does not eliminate the worm killing phenotype, merely reducing the rate of killing. Therefore it is not an essential virulence factor in the nematode[108].

Another battery of virulence factors possessed by *P. aeruginosa* are a variety of proteases, production of which is subject to regulation by LasR[109]. These are enzymes which hydrolyse peptide bonds within peptides and proteins[110]. Elastase B, a metalloprotease, is the product of the *lasB* gene[111] and is regulated by both the *las* and *rhl* QS systems[112]. Indeed, it was its role in elastase regulation that resulted in the discovery of the *lasR* gene[113]. It cleaves a variety of host organism proteins, including a variety of Immunoglobins and the structural proteins collagen and elastin[114]. Elastase A, a zinc metalloprotease, is coded by the *lasA* gene. This protease breaks down a number of glycine containing proteins[115]. It has also been shown to enhance the activity of Elastase B. The combined action of both these elastases, in addition to several other enzymes, is involved in the adoption of an invasive phenotype in several *P. aeruginosa* strains[115-118]. Elastase A has also been linked with the killing of *Staphylococcus aureus*, a common competitor of *P. aeruginosa* in the cystic fibrosis lung[119-121]. Alkaline protease is a zinc-dependent metalloendopeptidase[111]. It degrades laminin, a key component of the basal lamina[122]. This suggests a possible major role in invasion and haemorrhagic tissue necrosis[114]. Alkaline protease also inactivates a number of components of the host immune response, including neutrophils, human tumour necrosis factor α, γ-interferon and interleukin-6[123-126].

Regulation of rhamnolipid production is QS controlled, in fact it was the role of RhlR in the regulation of rhamnolipid biosynthesis that led to the initial identification of the *rhlR* gene[127]. Rhamnolipids are involved in *P. aeruginosa* motility phenotypes, playing an important role in both swarming and twitching motility[128, 129]. Additionally, rhamnolipids play a role in the spatial organisation of *P. aeruginosa* populations. Under the control of QS, rhamnolipids are responsible for the maintenance of water channels around *P. aeruginosa* microcolonies within biofilms[130]. However, they can also have a deleterious effect on the biofilm in enough numbers, as early overproduction of rhamnolipids has been shown to inhibit the initial formation of biofilms[130].

P. aeruginosa also produces exogenous polysaccharides. These are primarily used in the composition of biofilms[131]. Three polysaccharides are present in *P. aeruginosa*, Pel, Psl and alginate[132]. The *P. aeruginosa* genome retains the ability to synthesise all 3 polysaccharides[132]. However, any individual strain often only produces 1 at any specific time[133-135]. Alginate is the best studied of the 3, its primary role is making *P. aeruginosa* colonies mucoid[136]. It is mostly of importance in the cystic fibrosis lung. In fact it is only usually found in clinical isolates taken from CF pulmonary tissue[132](Franklin2011). Less is known about the remaining 2 polysaccharides. The presence of active Psl has been linked with the growth of biofilms[137]. Pel, on the other hand has been linked with spatial structuring within *P. aeruginosa* colonies, specifically in pellicles that form on stagnant cultures[134].

Biofilms are sedentary microbial communities. Cells adhere to surfaces and are supported by a structured matrix[131] of polysaccharides, extracellular DNA and proteins. The latter of which hold the biofilm together[138]. They are very common in bacterial infections, and are often found at infection sites[139]. In addition to colonising living organisms, biofilms can also grow on inorganic surfaces, bacterial biofilms growing on medical devices being a major cause of nosocomial infections[140]. As a social behaviour, biofilm formation is controlled by cell-cell communication, including QS. In *P. aeruginosa*, biofilms are under the control of C-di-GMP (which is itself potentially linked with the *lasI/R* system), as well as the *rhlI/R* and PQS QS systems[131]. The establishment of biofilms offers bacteria, including *P. aeruginosa*, a variety of ways to resist antimicrobial attack, both from administered drugs, and the immune system. These include limiting exposure to antimicrobials by acting as a physical barrier, the use of efflux pumps to remove antimicrobials which do penetrate the biofilm and the establishment of nutrient and oxygen gradients, which lower the metabolic rate of bacteria within the biofilm, reducing their susceptibility to antimicrobials. It has also been reported that biofilm phenotypes are linked with the emergence of persister variants and the phenotypic changes that result in the emergence of antimicrobial resistant variants of *P. aeruginosa*[141].

Project Aims

The trade-off hypothesis of virulence evolution, despite a substantial amount of theoretical and modelling work, remains relatively untested under *in vivo* conditions. The studies that have been performed have either utilised

macroscopic hosts and pathogens[142], or bacteria-phage interactions with a bacterial host[28, 143, 144]. There is a comparative lack of studies looking at bacteria as the pathogen. *P. aeruginosa* is a well-studied pathogen, that has already demonstrated varying levels of virulence by strain[145, 146], and is of clinical relevance. Therefore, this project was themed towards assessing the nature of *P. aeruginosa* virulence evolution using *in vivo* host models.

The specific aims were:

1. To determine the effect of *P. aeruginosa* strains on the nematode worm *Caenorhabditis elegans* (Chapter 2). This was done to test how different laboratory bacterial strains compare to isolates taken directly from clinical infections, and to test the roles of known virulence factors in the *C. elegans* model. Mutants lacking virulence factors displayed reduced virulence. The tested clinical isolates displayed virulence phenotypes more similar to those of laboratory strains originally taken from similar clinical conditions.
2. To perform a long-term selection experiment in a *C. elegans* host to test the role of host density (transmission rate) on virulence evolution, and to phenotypically and genomically characterise evolved bacteria (Chapter 3). It was found that varying host density did not significantly alter virulence levels, with controls displaying similar virulence phenotypes to worm evolved bacteria. Also, virulence proved to be a multifactoral process, with several virulence factors required to achieve full virulence in the *C. elegans* host.

3. To ascertain the extent of host adaption, and whether virulence and avirulence are conserved across multiple hosts. This was studied using 2 additional model organisms, *Galleria mellonella* (waxmoth larvae) and *Vigna radiate* (mung bean seedlings) (Chapter 4). Both models behaved very differently to the *C. elegans*. While the *C. elegans* host displayed a variety of *P. aeruginosa* virulence phenotypes, virulence was universally high in *G. mellonella* and low in *V. radiate*. So, the level of *P. aeruginosa* virulence is dependent in the host organism.

Chapter 2: Testing the virulence of laboratory and clinical *Pseudomonas aeruginosa* strains in *Caenorhabditis elegans*.

Abstract

Two types of bacterial strains are commonly used in research. Established laboratory strains, which have been used in research for years and can be wildly divergent from their wild roots, and strains recently isolated from environments, including infections. This raises the question, does time spent as a laboratory strain alter virulence levels and the ability to cause infection? To address this both laboratory and clinical strains of *P. aeruginosa* were tested for virulence towards the nematode worm *Caenorhabditis elegans*. Defined mutants lacking known virulence factors did display reduced virulence towards the nematodes, however, clinical isolates all displayed virulence phenotypes more similar to the PAO1 laboratory wild type and showed reduced virulence compared to the PA14 wild type. As the available clinical isolates were all taken from chronic infections, PAO1 was the laboratory strain with closest virulence effects to the isolates tested. These results suggest that laboratory strains retain virulence trends in line with their environmental origins, emphasising the need for careful selection of strain when designing studies.

Introduction

Understanding virulence, the capacity of a microbe to cause damage to a host, is key to understanding how pathogens cause disease, and in identifying potential new avenues for treatment[8]. Certain gene products have been identified and classified as virulence factors, such as the proteases produced exogenously by *P. aeruginosa*. Many of these virulence factors have been subjected to intense study of both the direct biosynthetic pathways and the regulatory networks responsible for their production and regulation[116, 147]. However, a great deal of this information has come from *in vitro* studies[100, 106, 116, 147-149] and there is now a great need for more *in vivo* studies. Until the cumulative effect of these "virulence determinants" are tested under *in vivo* conditions, rather than focussing on individual factors, it is difficult to draw robust conclusions about their true function. In addition, many studies use established laboratory strains, and defined mutants, and these are often assumed to reflect the clinical reality of specific organisms. The similarities and differences between laboratory strains and clinical isolates is an avenue rarely explored. Likewise, what *in vivo* studies there are tend to focus on a single example host organism, and do not address how virulence phenotypes may vary between different hosts.

There is a wide variety of *in vivo* host models available to the modern researcher, but they all have their pros and cons. A balance must often be found, taking into account cost, ethical considerations, ease of use, throughput of the model and (when dealing with human pathogens) clinical relevance. For example, some mouse models show similarities to human infections, but have high cost and high

skill requirements for use, both of which limit throughput, as well as ethical concerns constraining their use[150]. In this chapter, the focus was on the nematode worm *Caenorhabditis elegans,* its susceptibility to *P. aeruginosa* infection and factors potentially involved in virulence towards this organism.

C. elegans is one of the most commonly used organisms in laboratories throughout the world[151]. As a nematode worm, it occurs naturally in soil and leaf litter and is capable of inhabiting other small organisms in the soil[152, 153] and living freely on its own[154].

Originally introduced as a model organism in the 1970s to study development and neurobiology[155], *C. elegans* has since been used to study a wide range of biological processes, including ageing, apoptosis, cell cycle, cell polarity, cell signalling, gene regulation, metabolism and sex determination[156].

There are good reasons for the popularity of *C. elegans* in the laboratory. It is easy to maintain and use. Its rapid, hermaphroditic reproduction cycle allow for the rapid cultivation of substantial worm populations. These populations can be readily maintained and fed on *Escherichia coli* under basic laboratory conditions. The small size of the worm helps facilitate its use in assays involving a large number of individual organisms. *C. elegans* are transparent, which allow for the use of *in vivo* fluorescent markers to study individual processes[157], including a large number of disease genes and gene pathways[158]. The majority of known human signal transduction pathways are conserved within worms, with *C. elegans* possessing 12 of the 17 found in humans[159].

As a model host organism to measure bacterial virulence, various interactions between *C. elegans* and *P. aeruginosa* have been previously documented. *P. aeruginosa* is capable of exhibiting a number of phenotypes in *C. elegans*, depending on the environmental conditions. Low phosphate conditions have been linked to a rapid (fast) killing of *C. elegans*. This also causes a distinct red colouration of the worm's digestive tubes preceding death. This lethal phenotype has been linked with three virulence systems, phosphate signalling, pyoverdin and QS via the MvfR–PQS pathway[81]. Extremely rapid death via paralysis , occurring within 4 h of exposure via a mechanism controlled by the LasR and RhlR QS system has also been reported under rich conditions for bacterial growth[145]. Another fast killing phenotype has been observed under conditions of high osmolarity[108]. Subsequent work under these conditions has identified a trio of phenazines, including pyocyanin, which are highly toxic to the nematodes[160]. A low-nutrient environment, on the other hand, induces a 'slow kill' phenotype, in which the *C. elegans* are killed over the course of several days[161]. Both of these latter two phenotypes have been linked with the *las/rhl* QS system[161-163].However, this wide variety of virulent phenotypes are not universally present in all strains of *P. aeruginosa*. For example, PA14 lacks the ability to induce death via the lethal paralysis pathway, that PAO1 possesses[145].

The aim of this chapter was to test the virulence of multiple strains and mutants of *P. aeruginosa* in the *C. elegans* host model. These virulence phenotypes were then compared to clinical isolates tested in the same *C. elegans* model, to

determine whether virulence is similar between the clinical isolates, laboratory strains and defined mutants.

A number of well characterised *P. aeruginosa* mutants were tested, which have been previously linked with *P. aeruginosa* virulence. These included knockout mutants for all 3 major QS systems in *P. aeruginosa* (*las, rhl* and PQS), as well as knockout mutants for the PSL and PEL polysaccharides produced by *P. aeruginosa*. Multiple *P aeruginosa* wild type strains were tested, as these can display highly varied phenotypes, even within what is ostensibly the same strain[164]. This work was to support studies on the evolution of virulence (see Chapter 3), because differences in virulence due to phenotype in characterised mutants would indicate that different virulence levels of *P. aeruginosa* could potentially evolve over time in a *C. elegans* host. Furthermore, this could offer unique insights into what actually regulates virulence in a host if similar phenotypes to the mutants evolved. A selection of clinical isolates taken from cystic fibrosis lung sputum and diabetic chronic wound infections were also tested. This aimed to reveal any similarities between the naturally occurring clinical isolates and the artificial mutants created for the express purpose of laboratory study. The methodology used was derived from assay conditions previously established to test *C. elegans* killing under both fast and slow conditions[108, 161].

Materials and Methods

Strains used in this study

Strain	Description	Source
***Caenorhabditis elegans*:**		
N2 Bristol	Wild Type *C. elegans*.	The Caenorhabditis Genetics Centre, University of Minnesota[165].
***Escherichia coli*:**		
OP50	Uracil auxotroph. Used as food source for *C. elegans* and as a negative control. Tetracycline^{R} (Tc)	Epstein & Shakes (1995)[166]
***Pseudomonas aeruginosa*:**		
PA14	PA14 wild type.	Rahme et al (1995)[146]
PA14 Δ*lasR*	PA14 with a *lasR* insertion mutation.	Rumbaugh et al (2009)[167]
PA14 Δ*lasI*	PA14 with a *lasI* insertion mutation.	Rumbaugh et al (2009)[167]
PA14 Δ*rhlR*	PA14 with a *rhlR* insertion mutation.	Rumbaugh et al (2009)[167]
PA14 Δ*rhlI*	PA14 with a *rhlI* insertion mutation.	Rumbaugh et al (2009)[167]
PA14 Δ*pqsE*	PA14 with an unmarked *pqsE* deletion.	Steve Diggle Strain Collection, unpublished.
PA14 Δ*pqsR*	PA14 with an unmarked *pqsR* deletion.	Steve Diggle Strain Collection, unpublished.
PAO1-N	PAO1 Nottingham wild type.	The Holloway Collection
PAO1-N Δ*pqsA*	PAO1-N *pqsA* deletion mutant.	Aendekerk et al (2005)[168]
PAO1-N Δ*pqsE*	PAO1-N *pqsE* deletion mutant.	Rampioni et al (2010)[169]
PAO1-P	PAO1 USA wild type.	Holloway et al (1979)[170]

Strain	Description	Source
PAO1 P Δ*pelA*	PAO1-P *pelA* mutant, polar knockout of *pel* operon.	Borlee et al (2010)[171]
PAO1-P Δ*pslBCD*	PAO1-P *pslBCD* mutant, polar knockout of *psl* operon.	Kirisits et al (2005)[172]
PAO1-P Δ*pel/psl*	PAO1-P *pelA/pslBCD* double mutant.	Borlee et al (2010)[171]
CW2 B1	Clinical Isolate from Chronic Diabetic Ulcer Wound, taken from patient's bone.	Ana DeSilva, The University of Nottingham, Unpublished.
CW3 T1	Clinical Isolate from Chronic Diabetic Ulcer Wound, taken from patient's tissue.	Ana DeSilva, The University of Nottingham, Unpublished.
CW3 B1	Clinical Isolate from Chronic Diabetic Ulcer Wound, taken from patient's bone.	Ana DeSilva, The University of Nottingham, Unpublished.
CW4 T1	Clinical Isolate from Chronic Diabetic Ulcer Wound, taken from patient's tissue.	Ana DeSilva, The University of Nottingham, Unpublished.
CW5 T1	Clinical Isolate from Chronic Diabetic Ulcer Wound, taken from patient's tissue.	Ana DeSilva, The University of Nottingham, Unpublished.
CW5 T2	Clinical Isolate from Chronic Diabetic Ulcer Wound, taken from patient's tissue.	Ana DeSilva, The University of Nottingham, Unpublished.
SED2	Clinical Cystic Fibrosis Sputum Isolate Haplotype 2.	Darch et al (2015)[173]
SED6	Clinical Cystic Fibrosis Sputum Isolate Haplotype 6.	Darch et al (2015)[173]
SED8	Clinical Cystic Fibrosis Sputum Isolate Haplotype 8.	Darch et al (2015)[173]
SED16	Clinical Cystic Fibrosis Sputum Isolate Haplotype 16.	Darch et al (2015)[173]
SED20	Clinical Cystic Fibrosis Sputum Isolate	Darch et al

Strain	Description	Source
	Haplotype 20.	(2015)[173]
SED22	Clinical Cystic Fibrosis Sputum Isolate Haplotype 22.	Darch et al (2015)[173]

Table 2.1: Summary of bacterial strains and worms used in this Chapter.

C. elegans Husbandry

C. elegans were maintained on Nematode Growth Medium (NGM) agar plates (2.5 g l^{-1} Peptone, 3 g l^{-1} NaCl, 17 g l^{-1} agar (Oxoid LP0011), Cholesterol (5 mg ml^{-1} in ethanol), 1 ml l^{-1} 1M $CaCl_2$, 1 l^{-1} ml 1M $MgSO_4$ and 25ml l^{-1} 1M KH_2PO_4, the first three ingredients being added before autoclaving at 121°C, 15psi for 15 min, the remaining four ingredients were added after the sterilised media had cooled. The worms were fed on *Escherichia coli* (OP50 strain). To maintain a stock of adult worms, every 48-72 h a square of NGM agar was cut from an established *C. elegans* stock plate and transferred to a fresh NGM plate containing a lawn of OP50. Worms taken for assays, unless otherwise stated, were picked off stock plates using a length of flattened platinum wire under a Nikon SMZ1000 microscope at 2x magnification.

Worm Kill Assays

Both worm kill assays were adapted from protocols previously published[161].

Fast Kill Assay

Bacteria to be assayed were grown in liquid SK media (3.5 g l^{-1} Peptone, 3 g l^{-1} NaCl, 1 l^{-1} ml Cholesterol (5 mg ml^{-1} in ethanol), 1 ml l^{-1} 1M $CaCl_2$, 1 l^{-1} ml 1M

$MgSO_4$ and 25ml l^{-1} 1M KH_2PO_4) overnight at 22°C, 400 µl of this culture was then spread onto FK agar (10 g l^{-1} Peptone, 10 g l^{-1} NaCl, 10 g l^{-1} Glucose, 0.15M Sorbitol and 17 g l^{-1} agar) and incubated at 25°C for 24 h. Twenty *C. elegans* were then picked from stock plates onto the assay plate, which continued to be incubated at 25°C. The number of live *C. elegans* was counted every 12 h to assess the speed at which the *C. elegans* died under the high osmolarity of the assay conditions. Triplicate repeats were performed for each bacteria strain assayed.

Slow Kill Assay

Bacteria to be assayed were grown in liquid SK media overnight at 22°C, 400 µl of this culture was then spread onto SK agar and incubated at 25°C for 24 h. Twenty *C. elegans* were then picked from stock plates onto the assay plates, which continued to be incubated at 25°C. The number of live *C. elegans* was counted every 24 h to assess virulence levels. Triplicate repeats were performed for each bacteria assayed. If the worm density was so high that counting individuals was a practical impossibility, a value of 1000 was recorded.

Statistical Analysis

Statistical analysis of all acquired data was performed using Graphpad Prism 7, http://www.graphpad.com/scientific-software/prism/).

Results

Variations in temperature and bacterial growth media have little effect on the *C. elegans* killing phenotype

To assess the virulence of *P. aeruginosa* towards *C.* elegans, both fast and slow kill assays were performed. For the initial assays both SK and LB media were used, and the initial start-up cultures used to seed the assay plates with *P. aeruginosa* were grown at both 22°C and 37°C. This was done to determine whether the initial growth conditions of the bacteria have any effect on virulence towards *C. elegans*. By testing both of the killing assays under these varied initial conditions, any confounding effects of these on the seed culture could be discounted.

Previously published work grew *P. aeruginosa* using both rich growth media and high temperatures (most commonly LB media and 37°C), whereas more restricted growth conditions are associated with the *C. elegans* host (SK media and 22°C). The negative controls using the standard OP50 *C. elegans* food source resulted in the expected lack of virulence and worm killing under slow kill conditions. Fast kill conditions resulted in a restriction of *C. elegans* reproduction, as little growth in the worm population was observed during this assay (Figure 2.1A), however OP50 was not lethal to the *C. elegans*, even under the severe osmolarity conditions of the assay. Although the lack of worm killing was expected, the restriction of worm reproduction was not, as it has previously been reported that the fast kill conditions used here did not affect the growth and reproduction of *C. elegans* fed with OP50[108]. The only significant variation

in worm numbers was for SK 22°C, which was higher than the other three growth conditions (Tukey's multiple comparison test, all *p* values <0.02). However, even this spike in worm numbers, only at the 72 h time point, is very small compared to the degree of population growth observed under conditions of less severe osmolarity.

In contrast the slow kill assays resulted in rapid worm population growth, with all tested conditions reaching uncountable worm densities within 72 h (Figure 2.1B). While statistical analysis highlighted several significant variations, such as between grown on SK and LB, for both tested temperatures (Tukey's multiple comparison test, *p* values <0.001), all tested growth conditions reached the same level of worm saturation at the same time.

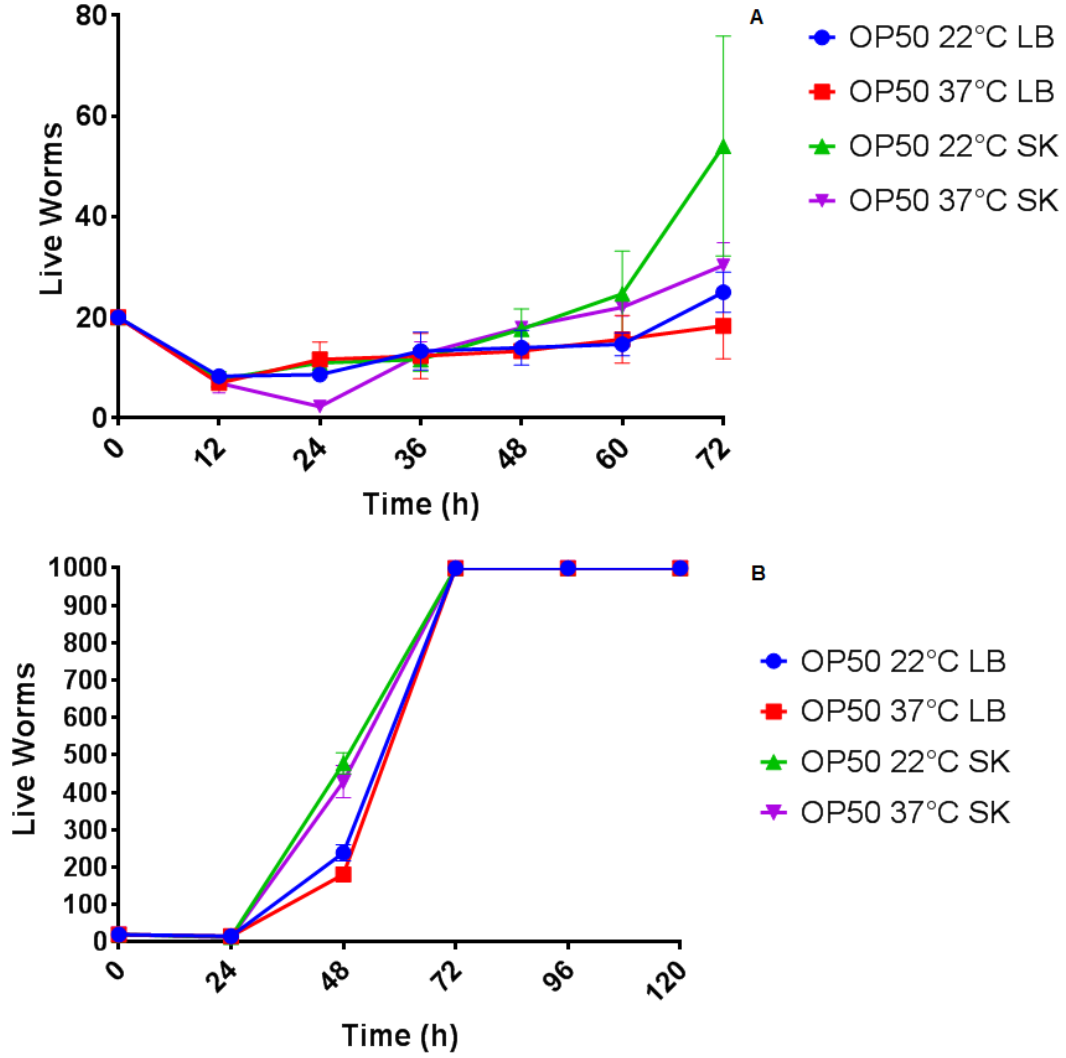


Figure 2.1: Worm killing assays of OP50, with initial culture grown in LB and SK media at 2 different temperatures for both the fast kill (A) and slow kill (B) assays. All results are shown as the means of three independent replicates, with the fast kill assay running for 72 h, and the slow kill assay 120 h. When error bars aren't visible, the bars are smaller than the symbols. The fast kill assay was run for a shorter time because 72 h was the point that all tested *P. aeruginosa* strains were 100% lethal to the worms. The fast kill assay restricted worm growth for all tested conditions, however no actual worm killing was observed. For the slow kill assay, there was no observed impediment to *C. elegans* survival and propagation. This results in levels of growth consistent with that observed on stock plates during routine maintenance of *C. elegans* stocks.

Having established the behaviour of *C. elegans* when exposed to an avirulent bacterial strain under the assay conditions, *P. aeruginosa* PA14 wildtype strain was then tested in both the fast kill and slow kill assays, with the same variations in initial seed culture media and temperature used for OP50. PA14 demonstrated a high level of lethality towards *C. elegans* in both sets of assays (Figure 2.2). This was most pronounced in the fast kill assay, with all growth conditions causing total worm extinction after 36 h exposure (Figure 2.2A). While the Slow Kill assay did not achieve total worm extinction, it still resulted in a high level of worm lethality, with only very small numbers of individuals persisting on the assay plates across the five days the assay ran (Figure 2.2B). Only the SK 22°C test showed a slight spike in worm numbers towards the end of the assay, the difference being statistically significant versus the other 3 growth conditions (Tukey's multiple comparison test, p values <0.176). Despite this difference, the SK 22°C growth conditions still demonstrated a large amount of virulence, as even the approximately 40 individuals present on these plates is a massive decrease when compared to the results seen when worms were grown on OP50 (Figure 2.1).

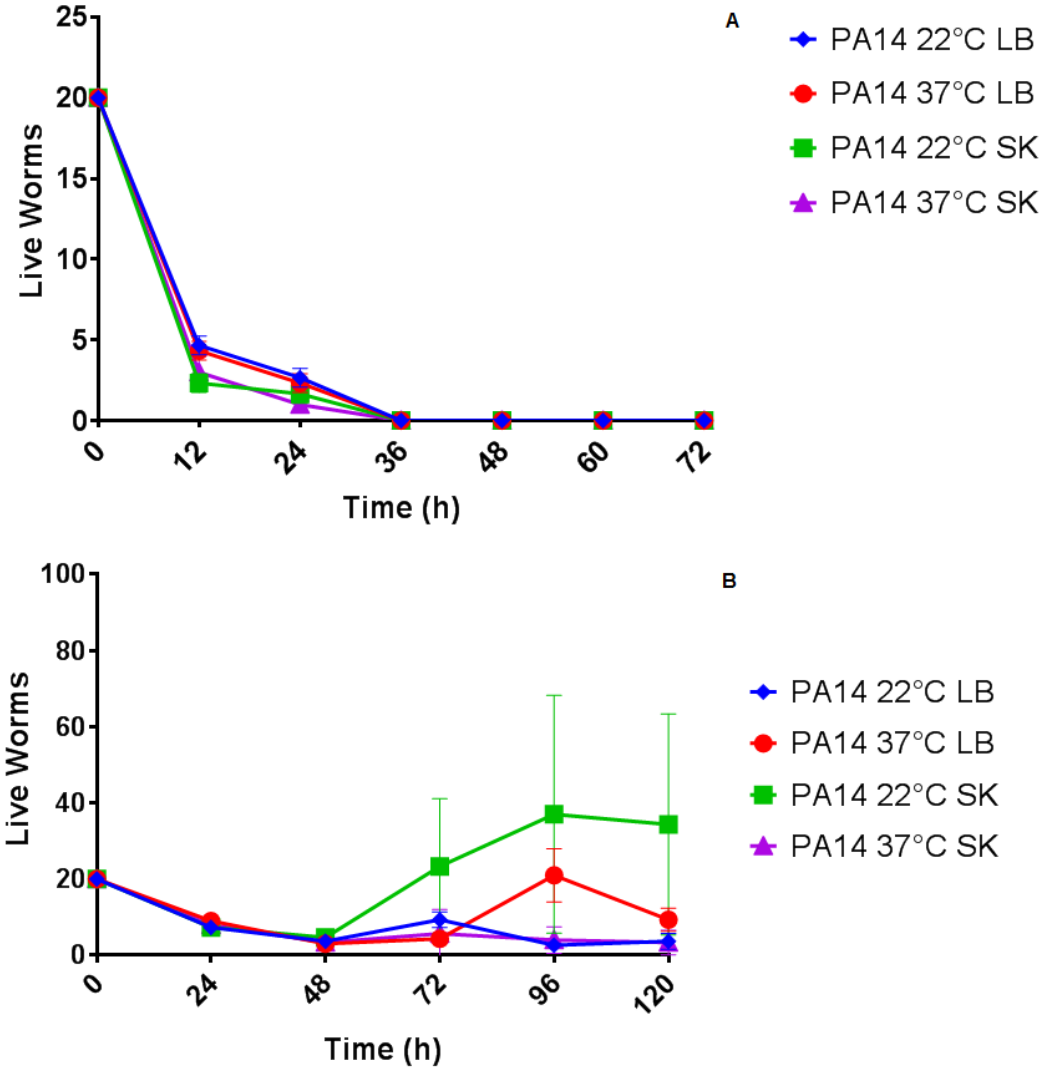


Figure 2.2: Worm killing assays of PA14, with initial culture grown in LB and SK media at 2 different temperatures for both the fast kill (A) and slow kill (B) assays. All results are shown as the means of three independent replicates, with the fast kill assay running for 72 h, and the slow kill assay 120 h. When error bars aren't visible, the bars are smaller than the symbols. Regardless of growth conditions, the fast kill assay induced rapid killing of *C. elegans*. The slow kill assay was highly virulent in three of the tested growth conditions. The 22°C SK conditions did show a slight recovery after 72 h, but overall *C. elegans* survival and propagation were still heavily reduced when compared with the avirulent OP50.

Quorum sensing is required for full *P. aeruginosa* PA14 virulence in *C. elegans* under fast kill conditions

Once the baseline virulence of the PA14 wildtype in *C. elegans* had been established, a variety of mutants previously shown to be altered in *P. aeruginosa* virulence were tested for *C. elegans* killing under fast kill and slow kill conditions. As the previous assays had not produced a difference in virulence between different seed culture growth conditions, the multiple conditions for the seed cultures was dispensed with. All subsequent fast kill assays used LB media and 37°C conditions for their seed cultures. Not all mutants tested were available in a PA14 background, so three different *P. aeruginosa* wildtype strains and their isogenic mutants were used. Mutants tested in a PA14 background were the QS mutants *ΔlasR, ΔlasI, ΔrhlR, ΔrhlI, ΔpqsE* and *ΔpqsR*. QS mutants tested in a PAO1 Nottingham background (PAO1-N) were *ΔpqsA* and *Δpqs*. Finally, several strains containing genes involved in exopolysaccharide biosynthesis, *ΔpelA, ΔpslBCD* and a *Δpel/psl* double mutant, were also assayed, along with their PAO1-P wildtype.

PA14 QS mutants all exhibited statistically significant reduced virulence compared to the wild type under fast kill conditions (Dunnett's multiple comparison test, p values <0.0001). While PA14 took 36 h to achieve 100% *C. elegans* mortality, all the tested mutants took at least twice as long, achieving 100% mortality by 72 h (Figure 2.3A). PAO1-N mutants did not demonstrate any variation in Fast Kill virulence, as both mutants and the wild type PAO1-N all achieved 100% worm mortality after 48 h (Figure 2.3B). This is slower killing than what was observed for PA14. The PAO1-P wildtype and its mutants displayed

similar behaviour to PA14, with little variation in mutants and wild type killing, and 100% worm mortality was achieved after 48 h (Figure 2.3C). The only exception was the *ΔpelA* mutant, which took 60 h to reach 100% worm mortality.

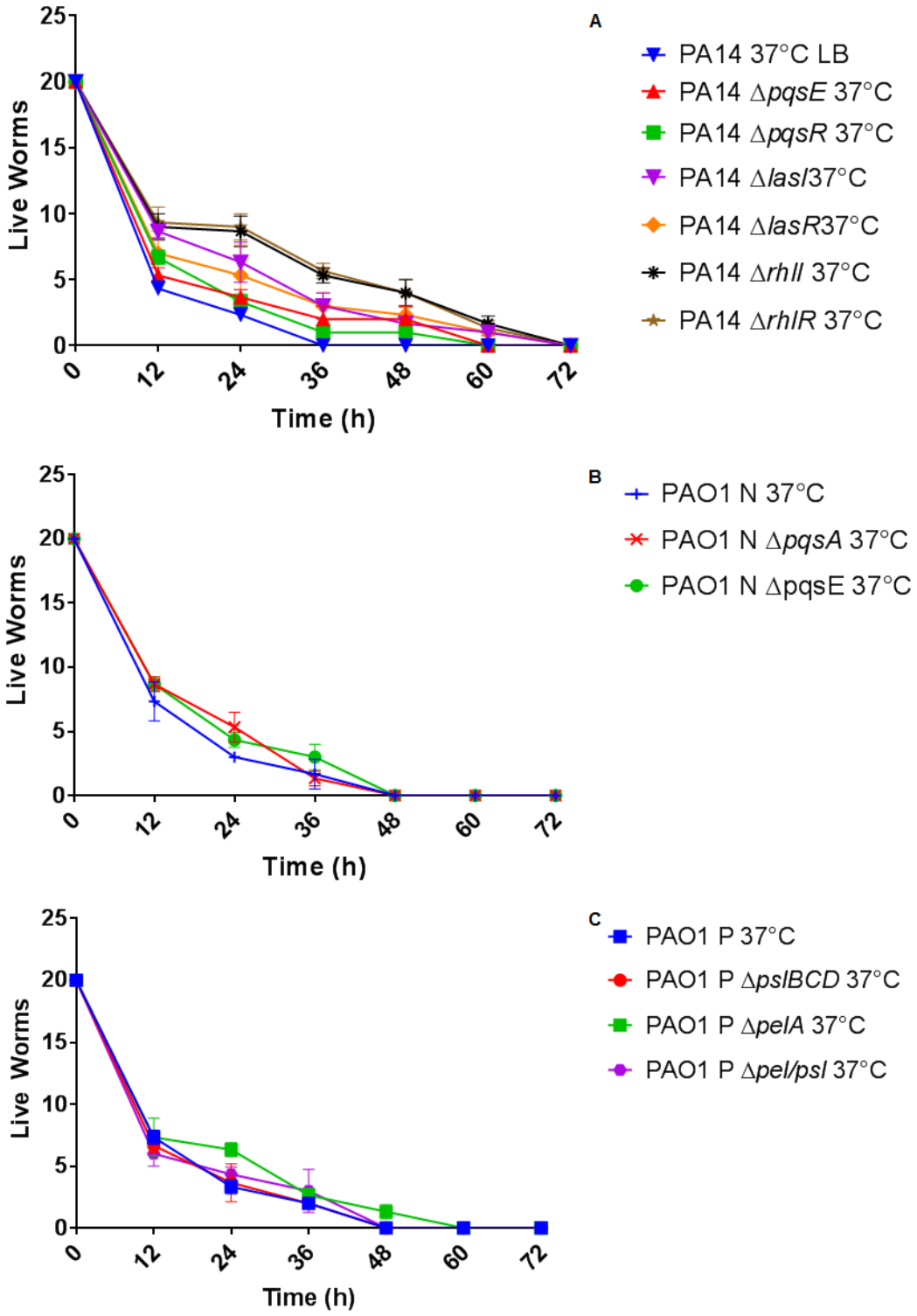


Figure 2.3: Fast kill assay of PA14 QS mutants (A), PAO1-N QS mutants (B) and PAO1-P polysaccharide mutants (C), with initial cultures grown in LB at 37°C. All results are shown as means of three independent replicates, over a period of 72 h. When error bars aren't visible, the bars are smaller than the symbols. All the PA14 QS mutants took longer to achieve 100% worm mortality than the wildtype. Both PAO1 strains, and their mutants were less virulent than PA14, but more virulent than the PA14 QS mutants. Nevertheless, all strains achieved 100% mortality within 72 h.

PA14 QS mutants and PAO1 strains display lower virulence under slow kill conditions than wildtype PA14

Slow kill assays were also performed on the same selection of *P. aeruginosa* mutants. For this set of assays the initial seed cultures were grown in liquid SK media (because this media was more relevant to the selection experiment in Chapter 3) at 22°C. However, the initial tests with PA14 on the slow kill assay did produce some differences between initial culture being grown at 22°C and 37°C (Figure 2.2). Therefore, the assay was also performed with seed cultures grown at 37°C, but there was no difference between these assays and the 22°C group (data not shown).

PA14 QS mutants displayed greatly reduced virulence under slow kill conditions compared to the wild type (Figure 2.4A). All results were statistically significant (Dunnett's multiple comparison test, p values <0.0001). All the mutants resulted in *C. elegans* populations which reached uncountable levels. Despite this eventual level of worm growth, there was some variation in the speed at which the QS mutants reached uncountable worm populations, with *ΔlasR* and *ΔlasI* taking only 72 h, while *ΔrhlI* and *ΔpqsE* took the full 120 h to reach the same point. The more striking result was in the behaviour of the respective wild types compared to PA14 (Figure 2.4). Both PAO1-N and PAO1-P exhibited an essentially avirluent worm killing phenotype under slow kill conditions. All PAO1 strains showed a very similar effect on the worms to the OP50 negative controls, with every strain resulting in an uncountable *C. elegans* density after 72 h.

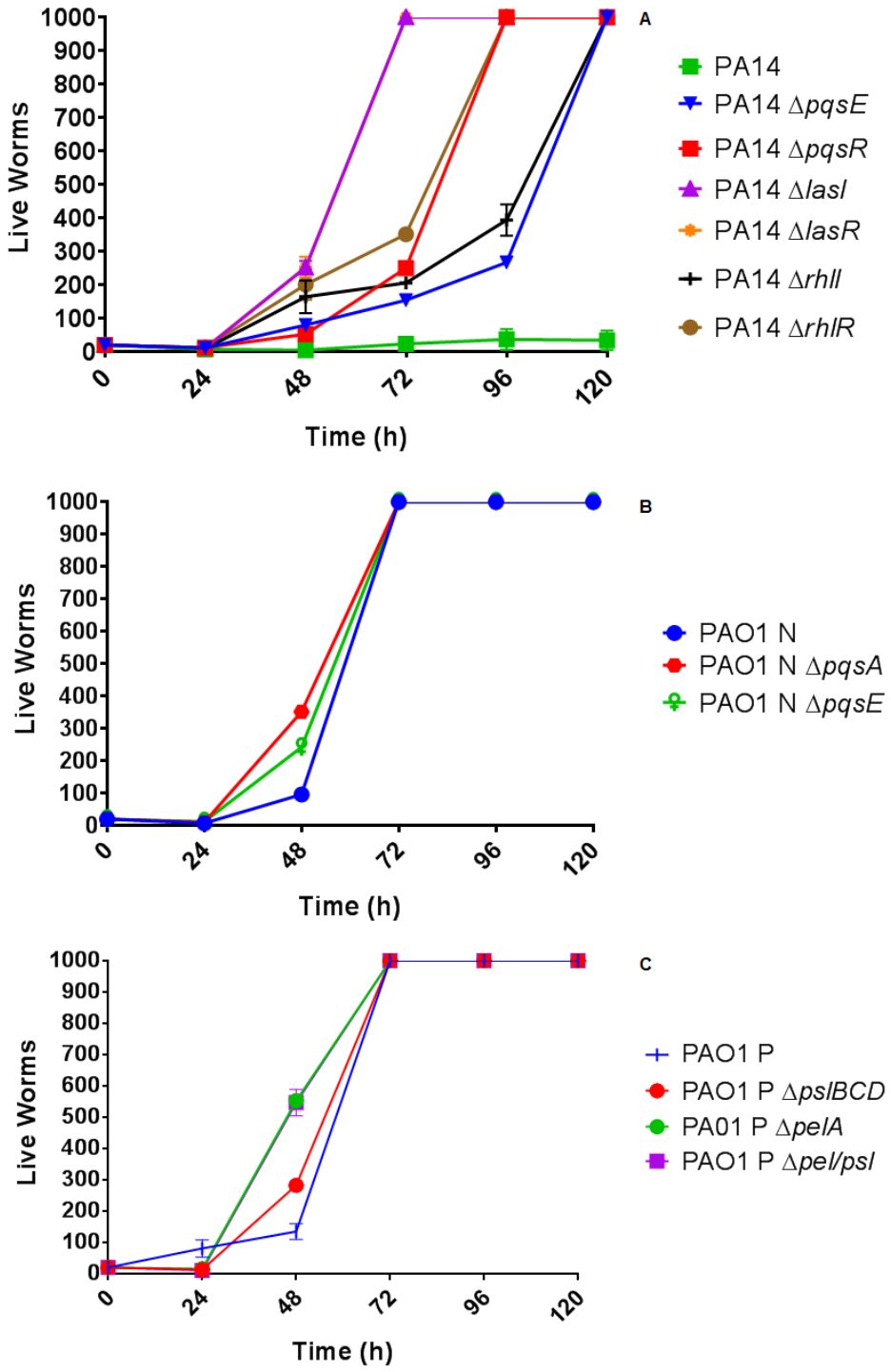


Figure 2.4: Slow kill assay of PA14 QS mutants (A), PAO1-N QS mutants (B) and PAO1-P exopolysaccharide mutants (C), with seed cultures grown in SK media at 22°C. All results are shown as means of three independent replicates, over a period of 120 h. When error bars aren't visible, the bars are smaller than the symbols. All PA14 mutants exhibit much lower levels of virulence than the wild type PA14, with all mutants reaching an uncountable *C. elegans* population. Every PAO1 mutant, as well as both wildtypes, all produced rapid worm growth, with all strains producing uncountable *C. elegans* populations after 72 h.

Virulence of *P. aeruginosa* clinical isolates under fast kill and slow kill conditions

To determine how similar virulence patterns of laboratory strains are compared to those isolated directly from infection, slow and fast kill assays were performed on a selection of clinical *P. aeruginosa* isolates: 6 isolated from the sputum of cystic fibrosis (CF) patients and 6 isolated from non-healing diabetic chronic wounds (CW).

Although the CF isolates all achieved 100% worm mortality in the fast kill assay within 72 h, there was some variation between the isolates (Figure 2.5A). The only isolate that displayed a significant (Tukey's multiple comparison test) difference was SED2, which displayed higher virulence than SED6 (p value 0.0118), SED16 (p value 0.0005) and SED22 (p value 0.001). No other comparisons between this set of isolates were statistically significant. SED2 was the most lethal of the isolates tested, achieving 100% worm mortality faster than any of the other tested isolates under fast kill conditions.

For the slow kill conditions, all tested CF isolates eventually reached uncountable worm populations (Figure 2.5B). However, 2 of the isolates differed in this assay, SED2 (less virulent than the other strains) and SED6 (more virulent than the other strains), both by statistically significant amounts (Tukey's multiple comparison test, all *p* values <0.0065). Interestingly, SED2 was the least virulent isolate under the slow kill conditions, maintaining the largest worm populations at the 48 and 72 h time points, in contrast to its behaviour as the most virulent isolate in the fast kill assay. SED6, on the other hand, was most virulent in this

assay than the other strains, taking the full 120 h to reach an uncountable worm population.

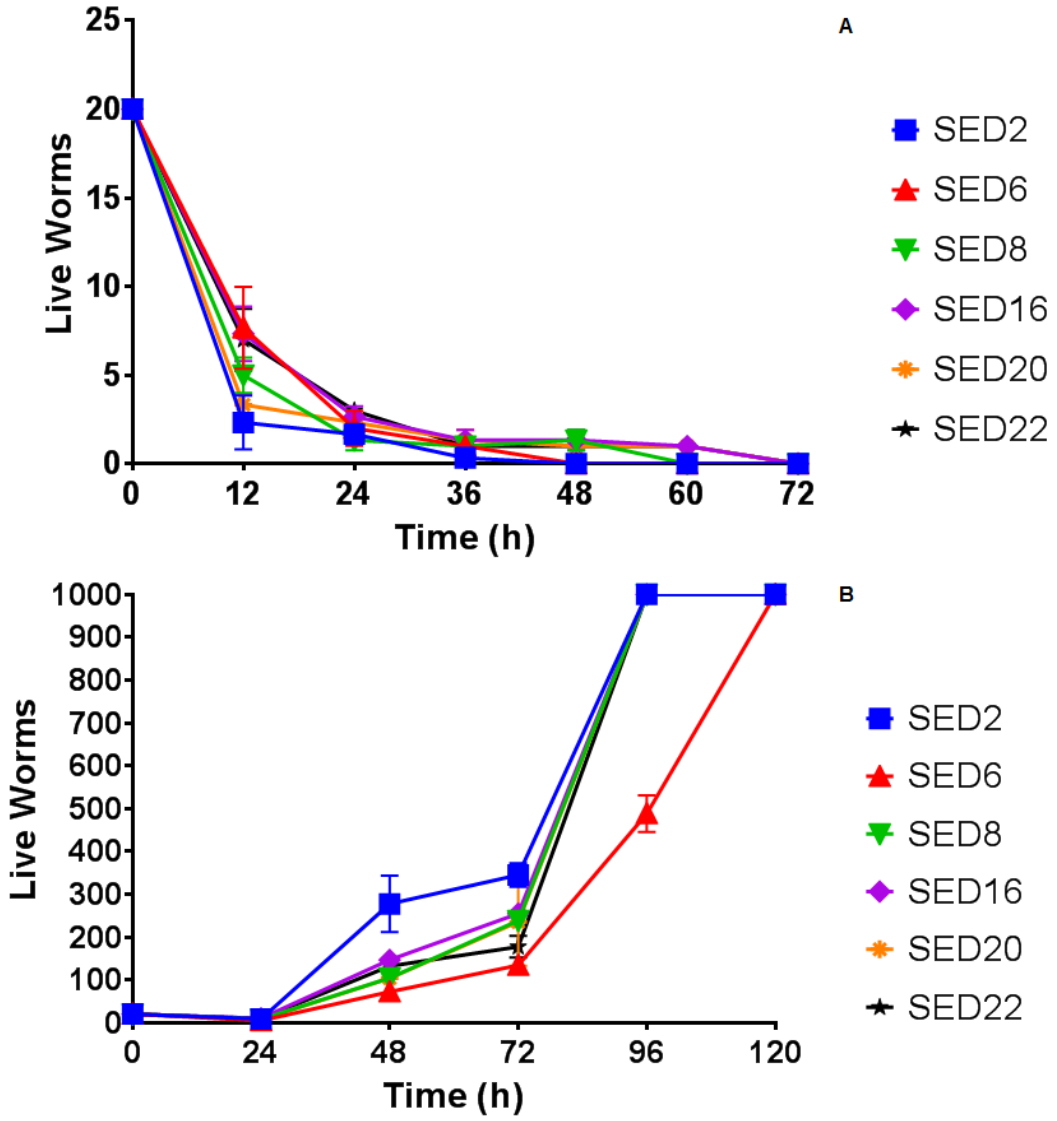


Figure 2.5: Worm kill assay of cystic fibrosis isolates. Initial cultures grown in LB media at 37°C. All results are shown as means of three independent replicates, over a period of 120 h. When error bars aren't visible, the bars are smaller than the symbols. For the fast kill assay (A), all isolates produced 100% *C. elegans* lethality after 72 h exposure. The slow kill assay (B) resulted in the majority of isolates reaching uncountable worm populations after 96 h, with only SED6 taking longer (120 h) to reach the same point.

The chronic wound (CW) isolates displayed similar virulence profiles to the CF isolates. For the fast kill assay, all isolates produced 100% *C. elegans* mortality between 36 and 72 h of exposure (Figure 2.6A). CW2 B1 was the only isolate to show a statistically significant difference compared to the rest of the group (Tukey's multiple comparison test, all *p* values <0.0001). This isolate was less virulent than the other strains, being the only tested isolate, which took the full 72 h to achieve 100% worm mortality.

In the slow kill assay, although all the isolates resulted in an uncountable worm density, a wide variety of virulence was observed between isolates (Figure 2.6B). There were significant differences between most of the isolates, with only the comparisons of CW2 B1 vs. CW3 B1, CW5 T1 vs. CW5 T2 and CW4 T1 vs. CW5 T1 and CW5 T2 not being significant (Tukey's multiple comparison test, all *p* values >0.1167). Of these isolates, CW3 T1 was the most virulent, taking the longest to reach uncountable worm density. CW5 T1 was the least virulent, producing the highest worm populations, and reaching uncountable density at the earliest time point, although 2 other isolates, CW4 T1 and CW5 T2, also reached uncountable worm populations at the 72 h time point.

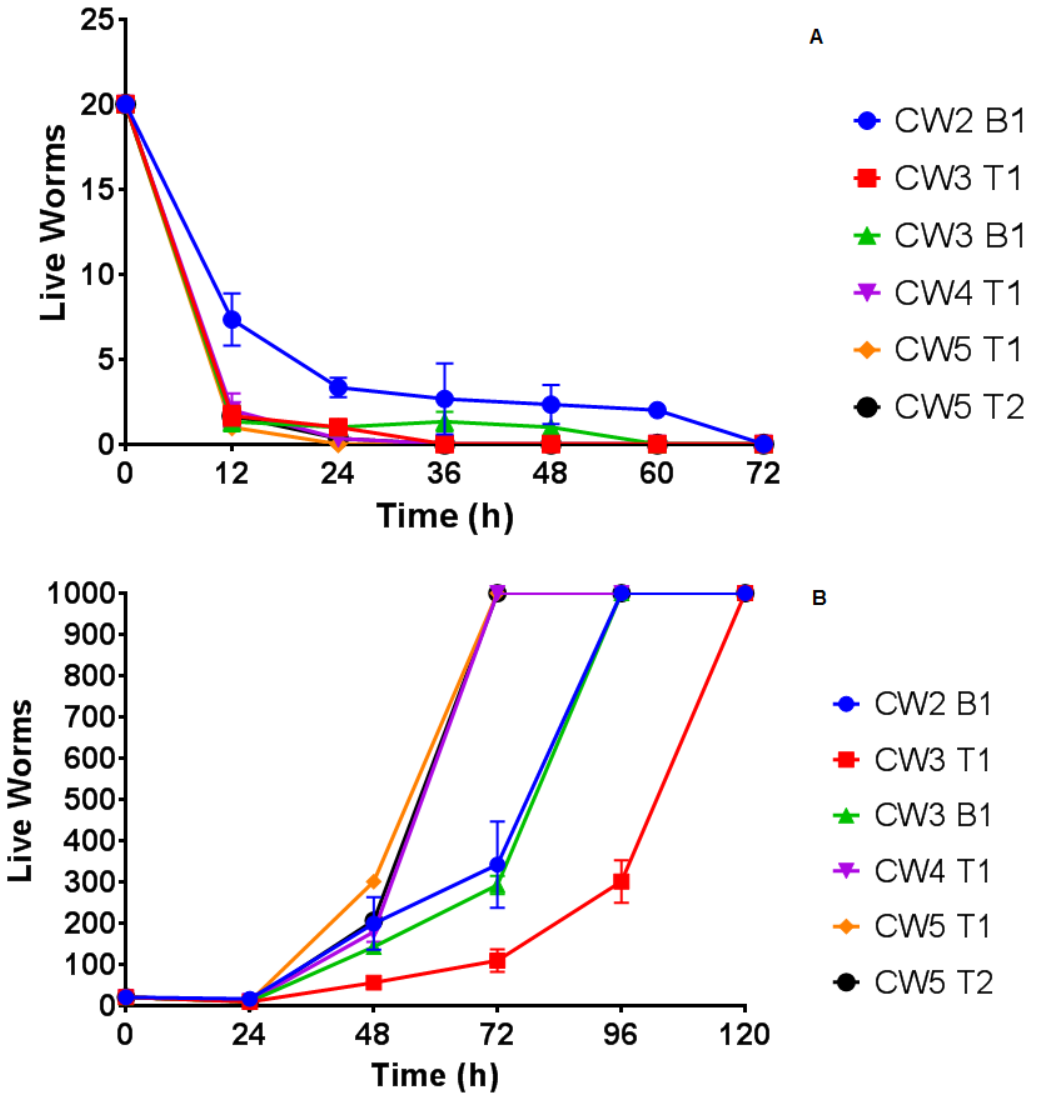


Figure 2.6: Worm kill assays of chronic wound isolates, initial cultures grown in LB media at 37°C. All results are shown as means of three independent replicates, over a period of 120 h. When error bars aren't visible, the bars are smaller than the symbols. For the fast kill assay (A), all isolates produced 100% *C. elegans* lethality after 72 h exposure. For the slow kill assay (B), 3 isolates (CW4 T1, CW5 T1 and CW5 T2) reached uncountable worm densities after 72h h exposure, 2 took 96 h (CW2 B1 and CW3 B1), while the last isolate (CW3 T1) took 120 h to reach that level of worm population.

The virulence profiles of CF and CW isolates are more similar to PAO1 and QS mutants than PA14

Most of the clinical isolates tested were less virulent than the PA14 wildtype in the fast kill assay (Figure 2.3). This was always the case for the cystic fibrosis isolates, the most virulent isolate, SED2, achieved 100% worm mortality after 48 h (Figure 2.5), while PA14 only needed 36 h. The chronic wound isolates had more virulent examples. Two isolates, CW3 T1 and CW4 T1, displayed similar virulence to PA14, achieving 100% mortality after 36 h, while CW5 T1 only needed 24 h (Figure 2.6). The remaining 3 isolates all took longer than PA14 to reach 100% worm mortality. So overall, 9 of the 12 clinical isolates tested had fast kill virulence levels closer to those of the PAO1 strains and the PA14 QS mutants, achieving 100% worm mortality in 48 to 72 h.

The similarities between PAO1 and the clinical isolates were even more pronounced in the slow kill assays. All the clinical isolates reached uncountable worm populations in this assay (Figures 2.5 and 2.6), just like both strains of PAO1, whereas PA14 remained the only tested strain that restricted worm population growth over the entire length of the assay (Figure 2.4).

Discussion

This chapter set out to demonstrate the varying levels of virulence that different *P. aeruginosa* strains, both laboratory and clinical, display in *C. elegans*. Virulence was tested under two established environmental conditions, one intended to promote rapid worm death, the other slower mortality.

The fast kill was always lethal to the worms, although some strains did take slightly longer to achieve complete lethality, rapid worm death occurred in all assays. The slow kill assays, on the other hand, were more varied. While there was a decrease in virulence for the PA14 QS mutants, the PAO1 mutants tested all displayed similar virulence profiles to their respective wildtypes. The attenuation observed in the *ΔlasI/R* and *ΔrhlI/R* mutants is consistent with other published studies[145, 161-163, 174]. PQS mutants, however, have not been tested in these conditions before, although PQS signaling has been implicated in the killing *C. elegans* under different environmental conditions[81]. However, the virulence levels for the PAO1 wildtypes were lower than that of the PA14 wildtype. Although there was some variation in virulence amongst the tested clinical isolates, overall, they displayed virulence profiles more similar to the PAO1 strains than PA14.

These data suggest that mutations in certain key gene pathways can have a dramatic effect on the virulence of *P. aeruginosa* towards *C. elegans*. As these major variations in virulence were all the result of single gene mutations, this suggests that major changes in *P. aeruginosa* virulence towards this host organism can easily arise after relatively few mutation events.

The results for both strains of PAO1 tested were surprising when compared with previous work on *C. elegans* and PAO1[81, 145, 162]. While PAO1 is known to be less virulent than PA14, at least some level of virulence under slow kill conditions was expected for PAO1. Given how even the less virulent PAO1 can be highly deadly to *C. elegans*[81], the extent of the difference observed between PA14 and PAO1 in slow kill conditions was unexpected. Unlike PA14, which remained virulent under these conditions, both PAO1-N and PAO1-P displayed a complete lack of virulence, achieving uncountably high *C. elegans* populations after only 72 h exposure, the same time it took for the avirulent OP50 food source. However, recent work using a different strain of PAO1, PAO1 Lausanne, showed lethal levels of virulence in *C. elegans*[5]. One possible explanation for this difference is that the avirulence seen here may be due to properties of the two specific PAO1 strains tested, rather than a general phenotype for the wide array of PAO1 strains that exist. Alternatively, the difference may be entirely due to variations in assay conditions, although the assays used here and in the previous study were broadly similar, so the severity of the difference observed is still surprising. However, if the latter is true, then this raises questions as to the suitability of the combination of PAO1 and *C. elegans* in research, if relatively minor changes in assay conditions result in such drastically different outcomes.

The lack of virulence displayed by the PAO1 mutants, given the avirulence seen in the wildtype PAO1 strains, was not surprising. However, this does not offer any insight into the affect these virulence factors may be have in virulent *P. aeruginosa* strains. The two *ΔpqsE* mutants tested highlight this, with the PAO1-

N mutant was avirulent, just like the wildtype, the PA14 mutant did present a reduction in virulence. However, none of the other PAO1 mutants tested were available in the PA14 strain. Therefore, exploring the effect of *pqsA* and the *pel/psl* polysaccharide biosynthesis genes in *P. aeruginosa* strains which are ordinarily virulent towards *C. elegans* remains beyond the scope of this study.

The PAO1 data does highlight the need for care when researching *P. aeruginosa*. PAO1 itself was first isolated in the 1950s[175], and has subsequently become the most commonly used laboratory strain for studying *P. aeruginosa*. After so many decades of laboratory use, the 'strain' PAO1 has been split into a variety of different strains, many more than the two tested in this Chapter[164, 175-177]. Indeed, the original PAO1 no longer exists in any known lab today. PA14, although a more recent strain, has still been in use for almost 20 years[146]. Therefore, there exists a wide variety of wildtype strains available for research purposes, all with unique sets of acquired mutations. Yet, data obtained with a particular PAO1 is often seen as directly comparable to data obtained with a different PAO1. Results reported in one strain will therefore often be reported as true for the species as a whole, despite the potential for high genetic and phenotypic divergence between strains. Therefore, there is a potential for confusion, as an observed phenotype may only occur due to a particular genotype unique to the studied strain. This concept has been highlighted in studies which examine the diversity of PAO1 genomes, especially with regards to the reproducibility of research[164]. This is a particular problem when publications do not specify the strain of PAO1 used[178-180]. The differences

between PA14 and PAO1 strains presented here, as well as the contrast of this PAO1 data with that of other published PAO1 strains[5], demonstrate that care must be taken when choosing the strain to research. What is true for one strain of *P. aeruginosa* cannot always be assumed to be true for another.

The CF clinical isolates all demonstrated broadly similar worm killing phenotypes, however virulence for some isolates did vary between slow and fast kill assay conditions. SED2 was amongst the most highly virulent isolates tested in the fast kill assay, with only a single individual surviving to the 36 h time point across all replicates. However, the opposite effect can be seen in the slow kill assay, with SED2 being one of the most benign towards the *C. elegans*. As SED2 has been shown to grow more rapidly than other similar CF isolates[173], this indicates that high bacterial population maybe an important factor in the fast killing phenotype of *P. aeruginosa*. Other isolates were more consistent, such as SED20 and SED22, which were amongst the least virulent CF isolates under both assay conditions. The similarities these two isolates displayed are despite substantial differences in pyocyanin production, which is highly produced in SED20, whereas SED22 is pyocyanin negative[173]. This indicates that pyocyanin may not be a key factor in *P. aeruginosa* virulence towards *C. elegans*, contrary to what has previously been reported[160]. However, the result was the same, regardless of virulence factor phenotype, avirulence under slow kill conditions. This loss of virulence is consistent with observed trends in chronic infections of the CF lung, although both virulent-sub populations and fully virulence-attenuated infections have been reported[181].

The Chronic Wound isolates behaved broadly similarly to the CF isolates under fast kill conditions, with rapid worm mortality. The two isolates which took the longest to achieve full mortality, CW2 B1 and CW3 B1, were both taken from bone samples, rather than tissue samples. While this in isolation would indicate that *P. aeruginosa* found in the bone expresses different phenotypes to that which persists in tissue, this correlation is not observed in the data from the slow kill assay, in which the bone isolates are within the range of the tissue derived samples. Therefore, if a specific phenotype is required for *P. aeruginosa* to persist in bone, it only influences virulence under very particular environmental conditions.

Just like SED2, CW4 T1 managed to be amongst the quickest killers for the fast kill assay, while having the largest countable worm population in the slow kill assay. This is in spite of having lower levels of virulence factors than the majority of the other CW isolates (DeSilva, Unpublished data). Just like with the CF isolates, this may indicate *C. elegans* death under fast kill conditions is dependent on factors other than the classic *P. aeruginosa* virulence factors.

When comparing the clinical strains to the laboratory strains, there was little difference in the results of the fast kill assays. Rapid inducement of worm death leading to 100% worm mortality occurred in all tested strains, regardless of origin. This additional data set further confirms the intrinsic high lethality of the fast kill assay conditions for *C. elegans*.

In the slow kill assays, both sets of clinical isolates displayed virulence phenotypes closer to those of the PAO1 strains, than PA14. The marked

difference in virulence observed can be explained by the fact that PA14 was isolated from a burn wound[182]. Burn wounds are acute infections where high virulence is an advantage to colonise and disseminate[183, 184]. PAO1, on the other hand, was derived from a chronic infection[175], and so a greater similarity in virulence to isolates with a similar clinical origin is to be expected.

This finding has the potential to impact on the choice of study organism. Instead of focussing on overall virulence levels of a strain, the focus should be on choosing a strain that is most similar in behaviour to those strains directly isolated from infections. For example, the data presented here suggests that using PA14 as a study organism for cystic fibrosis is a mistake, as the PA14 laboratory strain does not match the observed *P. aeruginosa* phenotype found in the lungs of cystic fibrosis patients. Of course, this isn't to say that PA14 doesn't have its place, both as a higher virulence strain for use in more theoretical studies, and when examining other forms of *P aeruginosa* infection, such as acute burn wounds, from which PA14 was initially derived[182].

In the case of the CF isolates, *P. aeruginosa* shifts towards one end of the trade-off equilibrium, favouring reduced virulence and a longer time in the host. A similar set of phenotypes was observed for the CW isolates. Although these came from three different patients (CW4 and CW5 isolates being taken at different time points) (DeSilva, Unpublished data), they still indicate a similar evolutionary outcome to the CF isolates, which is unsurprising, given the chronic nature of both infections. Despite the virulent reputation of *P aeruginosa*[185],

these results suggest it will quite readily adopt attenuated virulence under conditions of chronic infection.

In summary, this study replicates the previously observed high lethality of *P. aeruginosa* towards *C. elegans* under high osmolarity 'fast kill' conditions, regardless of the *P. aeruginosa* strain used. The less severe 'slow kill' conditions, on the other hand, provided a more varied array of virulence phenotypes. Reduced virulence in multiple *P. aeruginosa* PA14 virulence factor mutants was observed, demonstrating that relatively few mutations in the right places have the potential to drastically alter *P. aeruginosa* virulence. However, this was only the case for PA14 mutants, as both PAO1 wildtype strains tested displayed similar virulence profiles to their respective mutants. Furthermore, the clinical *P. aeruginosa* strains tested demonstrated more phenotypic similarity to the PAO1 strains than to PA14. Therefore, PAO1 may more closely represent the virulence of *P. aeruginosa* in the clinical context of cystic fibrosis lungs and non-healing diabetic wounds, rather than the more virulent PA14.

Chapter 3: Studying the Evolution of Virulence in *Pseudomonas aeruginosa* Populations Evolved in Varying Host Densities

Abstract

Previous work studying virulence evolution has traditionally focussed on either macroscopic host-parasite systems, such as nematodes parasitising fig wasps, or bacteria-phage interactions where the bacteria are the host. There has been a relative lack of empirical work examining how virulence evolves when bacteria are treated as the pathogen. To investigate this, and the effect of host density and transmission on *Pseudomonas aeruginosa* virulence evolution, a novel experimental protocol was developed, where the highly virulent *P. aeruginosa* PA14 strain was repeatedly passed through a *Caenorhabditis elegans* host at both high and low host densities (theoretically relatively high and low transmission rates respectively). After 60 days of within host evolution, the resulting evolved populations, and individuals taken from populations, were assayed for a range of *P. aeruginosa* virulence factors, as well as overall virulence towards *C. elegans*. Although the selection experiment was successful in generating evolved phenotypes of *P. aeruginosa*, variation of virulence between bacteria evolved in high and low host density environments was not observed. Additionally, the evolved populations underwent whole genome sequencing to identify the number and location of SNPs present. Genomic changes did not correlate with phenotypic variation, meaning that the genetic underpinning of the evolved virulence changes could not be determined.

Introduction

The trade-off hypothesis was put forward to explain the wildly divergent nature of virulence between different pathogens, which can range from inconveniencing the host (such as Rhinovirus[41]), to being highly lethal (such as Ebola[25]). Even strains of the same species can display substantial variations in virulence, as seen in the differences in virulence between the classical and El Tor strains of *Vibrio cholerae*[40] and PA14 and PAO1 in *P. aeruginosa*[183, 184]. The trade-off hypothesis states that there is an inverse relationship between the window of opportunity for transmission and virulence, so that higher virulence will degrade the host faster and shorten the time available to spread to fresh hosts[18, 32, 34]. The trade-off between these factors results in a unique optimal level of virulence for each pathogen, where host degradation and transmission are balanced[35, 36]. However, this equilibrium point is the result of host-pathogen interactions under particular environmental pressures at a specific time. Therefore, a change in those environmental pressures, such as alterations in host availability, should theoretically shift the equilibrium point of the trade-off and result in the evolution of altered virulence.

In addition to this basic framework for viewing the emergence of virulence, the very nature of what qualifies as a pathogen has recently been called into question. In a similar way to how the trade-off hypothesis predicts a unique equilibrium point of optimal virulence for each host-pathogen interaction environment, this has been extended to theorising that all pathogenicity is due to the specific way a host interacts with any given microbe[7, 8, 10]. Under this

concept, there is no such thing as an inherent pathogen and pathogenicity and virulence can only occur if the correct set of microbe-host interactions take place[8]. This way of viewing the emergence of disease is particularly persuasive due to the lack of any structure or function that is unique to 'pathogens', identified virulence factors are found in non-pathogenic strains of microbe[10]. The opposite has also been reported, with simple virulence factor availability not being a reliable predictor of virulence[186]. Just as with the trade-off hypothesis, changing environmental factors which impact the nature of the host-microbe interactions, such as host availability, should result in different levels of observed virulence.

The aim of this Chapter was to test the trade-off hypothesis in a *C. elegans-P. aeruginosa* host-pathogen model. Specifically, the goal was to test whether changing the environmental host density, and therefore transmission rate, could shift the equilibrium point of the trade-off. The working hypothesis was that a lower host density would provide evolutionary pressures to shift the equilibrium point towards longer infection times and reduced virulence. Therefore, when host density was low and chances of transmission more limited, bacteria would more quickly evolve to a reduced virulence state than bacteria evolved in high host densities where transmission could more readily occur.

Once the *P. aeruginosa* PA14 ancestor strain had undergone 20 rounds of selection, the evolved populations of bacteria were assessed for the production of known virulence factors and quorum sensing signal molecules. In addition, overall virulence towards *C. elegans* was assessed, to gauge the extent to which

virulence altered in evolved populations. The populations were genome sequenced, with the intention of tying changes in virulence to specific SNPs and genomic changes. Individual isolates were also taken from each population, and tested for the same virulence factors and virulence against *C. elegans*. This was to provide insights into the degree of heterogeneity and diversity in the evolved populations.

Materials and Methods

Strains used in this study

Strain	Description	Source
Caenorhabditis elegans		
N2 Bristol	Wild Type *C. elegans*.	The Caenorhabditis Genetics Centre, University of Minnesota[165].
Pharynx-tagged	*C. elegans* modified to constitutively express GFP in the pharynx.	Gifted to the Steve Atkinson strain collection
Escherichia coli		
OP50	Uracil auxotroph. Used as food source for *C. elegans* and as a negative control. Tetracycline^{R} (Tc).	Epstein & Shakes (1995)[166]
pSB536	JM109 strain containing AHL reporter plasmid to detect C4-HSL. Plasmid contained AHL biosensor, *ahyRI''::luxCDABE* in pAHP13. Ampicillin^{R} (Amp).	Swift et al (1997)[187]
pSB1075	S17-1 strain containing AHL reporter plasmid to detect 3-oxo-C12-HSL. Plasmid contained *P. aeruginosa* *lasR* gene and *lasI::luxCDABE* from *Photorhabdus luminescens.* Tc^{R}.	Winson et al (1998)[188]
Pseudomonas aeruginosa		
PA14	PA14 wild type.	Rahme et al (1995)[146]
PA14R	PA14 containing pME6032 plasmid encoding an mCherry fluorescent protein.	Roman Popat, Unpublished.
AN1	PA14 wild type containing a *lasB:lux* promoter fusion. Used as the ancestor strain for the selection experiment.	This Study

Strain	Description	Source
Evolved *P. aeruginosa*	Selection of heterogenous populations and individual isolates evolved during this study. A full list is provided in Appendix 1.	This Study

Table 3.1: Summary of bacterial strains and worms used in this Chapter.

C. elegans husbandry

C. elegans were maintained on Nematode Growth Medium (NGM) agar plates (2.5 g l^{-1} Peptone, 3 g l^{-1} NaCl, 17 g l^{-1} agar (Oxoid LP0011), Cholesterol (5 mg ml^{-1} in ethanol), 1 ml l^{-1} 1M $CaCl_2$, 1 l^{-1} ml 1M $MgSO_4$ and 25ml l^{-1} 1M KH_2PO_4, the first three ingredients being added before autoclaving at 121°C, 15psi for 15 minutes, the remaining four ingredients were added after the sterilised media had cooled). The worms were fed on *Escherichia coli* (OP50 strain). To maintain a stock of adult worms, every 48-72 h a square of NGM agar was cut from an established *C. elegans* stock plate and transferred to a fresh NGM plate containing a lawn of OP50. Worms taken for assays, unless otherwise stated, were picked off stock plates using a length of flattened platinum wire under a Nikon SMZ1000 microscope at 2x magnification.

Serial passage experiment

C. elegans was chosen as the host organism due to the ubiquity and ease of use of *C. elegans* as a model system[151] and its previous use with *P. aeruginosa*[5, 81, 104, 160, 161, 174]. Initial infection of *C. elegans* with *P. aeruginosa* took place on slow kill (SK) agar (3.5 g l^{-1} Peptone, 3 g l^{-1} NaCl, 17 g l^{-1} agar, 1 l^{-1} ml Cholesterol (5 mg ml^{-1} in ethanol), 1 ml l^{-1} 1M $CaCl_2$, 1 l^{-1} ml 1M $MgSO_4$ and 25ml l^{-1} 1M KH_2PO_4) spread with a relevant strain of *P. aeruginosa* taken from an overnight culture. A square of worm rich NGM from a *C. elegans* stock plate was then added to the plate and incubated at 22°C for 48 h. *C. elegans* from these infection plates were then used as the infected population in trial transmission assays.

At each round of passage, 15 *P. aeruginosa* infected *C. elegans* worms were taken from plates containing *P. aeruginosa* and placed on one side of a fresh bacteria-free plate, within 1 cm of the plate edge. Then 15 worms, not infected with *P. aeruginosa*, were taken from an OP50 stock plate and added to the other side of the assay plate, again within 1 cm of the plate edge. These plates were then incubated for 72 h at 22°C which allowed worms to fully disperse across the plate. This dispersal and mixing of the populations provided the opportunity for *P. aeruginosa,* excreted by the infected worms, to be consumed by uninfected individuals, transmitting the infection (Fig 3.1).

In the finalised long-term selection experiment, these plates were then used to provide the infected *C. elegans* population, containing the evolving *P. aeruginosa*, for the next round of selection. Control plates (without worms) were streaked with bacteria in the same area as the starting infected worm populations; this was subcultured onto fresh plates at the same time as a new round of selection. This process was repeated for 20 rounds of selection, using six repeats for each condition (high host density worm-evolved, high host density control, low host density worm-evolved and low host density control). Every 5 rounds of selection, 20 *C. elegans* were removed into 100 µl M9 buffer, killed by centrifugation (20238 rcf) and spread onto SK agar to collect the worm evolved bacterial populations. The evolved bacterial populations were then frozen at -80°C to preserve them for further analysis.

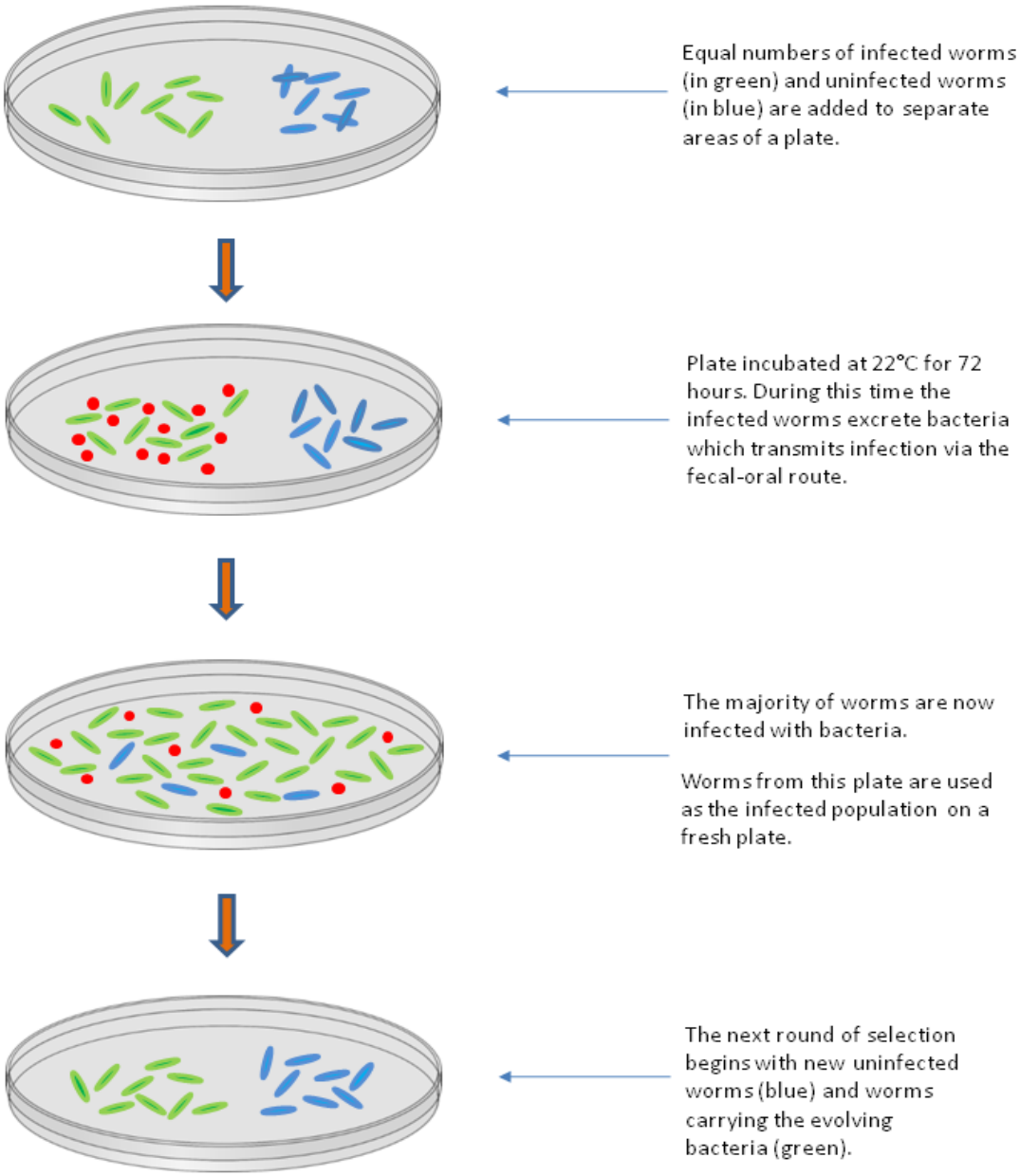


Figure 3.1: *C. elegans* and *P. aeruginosa* serial passage selection experiment regime. The experiment was initiated with the same culture of *P. aeruginosa* AN1. Host density was varied by using the same number of worms on plates of different sizes (168 mm plate diameter for low host density condition, 54 mm diameter for high host density conditions). Six replicate lines for each environmental condition, high host density worm evolved, high host density control, low host density worm evolved and low host density control, were started, although some of them did not survive the entire selection experiment. Control plates (no worms) were subcultured manually, with bacteria placed in the same region as the infected worms. Pictured here is one full round of passage. This cycle was performed 20 times during the selection experiment.

Extracting individual isolates from evolved populations

The evolved bacterial populations were grown overnight in liquid SK media at 22°C. These cultures were then diluted to a concentration of 10^{-6}. These dilutions were then plated onto SK agar and incubated at 22°C to generate single colonies. Congo Red Agar base was made (1 g l^{-1} Yeast Extract, 1 g l^{-1} Casamino Acids10 g l^{-1} agar) and autoclaved. To make the final Congo Red Agar, 2.5 mM $MgCl_2$, 100 µM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, 4 mM $CaCl_2$, 40 mg l^{-1} Congo Red, 0.4% glycerol and 1X M63 (10 mM KH_2PO_4, 1.5 mM $(NH_4)2.SO_4$ and 1.7 µM $FeSO_{3.6}H_2O$) were added to the autoclaved base. Single colonies from the SK plates were then picked onto the Congo Red agar and incubated at 37°C for 24 h. This was followed by 96 h incubation at 22°C. After the second incubation, the plates were observed for different colony morphologies. For each population, sixty colonies were picked onto the Congo Red, from these, 6 colonies were then chosen as the evolved isolates, attempting to cover as many varied morphologies as possible.

Visualising Worm Infections

To establish that worms were infected with *P. aeruginosa* during the long term selection experiment, worms were viewed under a Zeiss LSM700 microscope to ascertain the presence of fluorescent PA14 (PA14R) in the worm's digestive tract. To achieve this, worms were picked off the assay plates and placed into 10 µl sodium azide solution (400 mM) on a microscope slide to paralyse them. Once paralysed (after 1-2 minutes) fluoromount was added to the side to fix the worms in place. Once dry, a cover slip was applied and the slides were ready to be viewed. If infected, red fluorescence could be seen in the digestive tracts of

the *C. elegans*. Worms were viewed using a Nikon Eclipse 50i microscope with fluorescence intensifier.

Determining transmission of infection between worms

In addition to observing fluorescent *P. aeruginosa* in the worm digestive tract, a method was also required to ensure that any infection observed was the result of transmission between worms, rather than merely carry over from the initial infected population. To achieve this, two types of *C. elegans* worm were used, the N2 wild type, and worms in which the pharynx was constitutively expressing GFP (Pharynx-tagged). One of these strains was used as the infected group for each assay, with the second providing the uninfected population. Therefore, any worms displaying infection from the initially uninfected phenotype had to be the result of transmission, rather than individuals from the initial group of infected worms. The difference between wild type and Pharynx-tagged *C. elegans* was revealed by viewing worms using a Nikon Eclipse 50i microscope with fluorescence intensifier, under which the GFP tagged structures in the worms were readily visible.

To provide different worm densities between worms two different sizes of Petri dish were used. Small Petri dishes (54 mm diameter) were used for the high population density conditions, while larger dishes (168 mm diameter) were used for the low host density conditions.

Once the conditions of transmission had been established, the use of PA14 R and Pharynx-tagged *C. elegans* were discontinued for the main selection experiment,

to avoid unforeseen variables from the fluorescent proteins influencing the result.

To remove the high 'external' *P. aeruginosa* populations from the worm surface, Streptomycin (Sm) was utilised. *C. elegans* were washed off an infection plate with M9 buffer. Sm 200 µg/ml was then added to the buffer, shaken and left for 10 minutes at room temperature. This mixture was then added to a clean SK agar plate and air dried to facilitate worm picking. Twenty adult *C. elegans* were then picked onto fresh SK agar and incubated at 22°C. The level of bacterial growth was then compared with 2 controls; 20 worms picked directly from infection plates and 20 worms which underwent the M9 removal procedure, but without the Sm wash.

Slow kill *C. elegans* assay

The method used was adapted from one previously published[161]. Bacteria to be assayed were grown in liquid SK media overnight at 22°C, 400 µl of this culture was then spread onto SK agar and incubated at 25°C for 24 h. Twenty *C. elegans* were then picked from stock plates onto the assay plates, which continued to be incubated at 25°C. The number of live *C. elegans* was counted every 24 hours to assess virulence levels. Triplicate repeats were performed for each strain of bacteria assayed. If the worm density was so high that counting individuals was a practical impossibility, a value of 1000 was recorded.

Pyocyanin assay

P. aeruginosa strains were incubated overnight at 22°C in 2 ml CAMHB (21 g l^{-1} Mueller Hinton Broth, 20-25 mg l^{-1} 1M $CaCl_2$, 10-12.5 mg l^{-1} $MgCl_2$) media. 50 µl of this culture was then used to inoculate 5 ml of fresh CAMHB and incubated at 22°C for a further 16 h. Cells were pelleted by centrifugation at 1664 rcf for 30 min and the supernatant removed and filter sterilised using a 0.2 µm filter. 500 µl of the filtered supernatant was then mixed with 300 µl chloroform and vortexed until the mixture became cloudy. The separation of the chloroform and aqueous elements was facilitated by centrifugation at 15871 rcf for 2 min. The bottom chloroform layer was then extracted, another 300 µl was added and the vortexing and centrifugation steps repeated. Both chloroform extractions were then combined together. 250 µl of 0.2M HCl was added to the chloroform and the vortexing and centrifugation sequence repeated again. The HCl layer (pink in colour if pyocyanin is present) was then removed and absorbance at A_{520} was then measured on a TECAN Infinite M200Pro to determine pyocyanin levels.

Siderophore assay

P. aeruginosa strains were grown in Casamino Acid media (5 g l^{-1} Casamino Acids, 0.25 g l^{-1} $MgSO_4$, 0.9 g l^{-1} K_2HPO_4) at 22°C overnight. Cells were pelleted by centrifugation at 1664 rcf for 30 min. The supernatant was then filter sterilised using a 0.2 µm filter and 200 µl aliquots transferred to a UV sterilised 96-well plate. The filtered supernatant was then assayed for pyoverdin using an excitation wavelength of 400 nm and an emission wavelength of 460 nm. For pyochelin an excitation wavelength of 350 nm and emission wavelength of

430nm were used. A TECAN Infinite M200Pro was used to measure both siderophores.

Protease assay

P. aeruginosa strains were cultured overnight at 22°C in SK media. Cells were pelleted by centrifugation at 1664 rcf for 30 min and the supernatant was filter sterilised using 0.2 µm filters. 10 µl of the sterilised supernatant was then spotted onto skimmed milk agar plates, allowed to air dry and incubated at 22°C for 24 h. Protease activity was then assessed by the presence of a clearance zone on the plates where the milk proteins had been degraded. Proteinase K (5 mg/ml) was used as a positive control, and fresh LB media was the negative control.

Cyanide assay

Cyanide detection paper was made by soaking Whatman filter paper with detection solution, allowed to air dry, and was then cut to fit over the wells in a 96-well plate. The detection solution consisted of copper(II) ethylacetate (5 mg) and 4,4'-Methylenebis (N,N-dimtheylaniline) (5 mg) dissolved in chloroform (2 ml). Overnight culture of the bacteria to be tested (grown in liquid SK at 22°C) were standardised to a 1 in 50 dilution of OD_{600} 1.0. Triplicates of 250 µl or this diluted culture were added to the 96-well plate and the detection paper placed on top. To secure the paper a pre-sterilised breathable sealing film was placed on top of the plate and paper. A plate roller was used to ensure a tight and uniform fit. The plates then underwent static incubation for 15 h at 22°C. After the

incubation, the detection paper was examined, if cyanide was present a blue colour was observed on the detection paper.

Detection of *lasB::lux* promoter fusion

P. aeruginosa strains were grown overnight in SK media. Assay samples were made by adding 7 µl of overnight culture to 193 µl fresh SK media. These samples were then loaded into 96 well plates. The plate was then read on a TECAN machine. The OD_{600} and relative light output were read every 30 minutes over the course of an 18 hour assay.

AHL bioreporter assay

Cultures of the bacteria to be tested were grown in liquid SK at 22°C overnight. The bacteria were pelleted by centrifugation at 1664 rcf for 30 minutes, then the supernatants were filter sterilised using 0.2 µm filters. Overnight cultures of *E. coli* containing either the C4-HSL bioreporter plasmid pSB536 or the 3-oxo-C12-HSL bioreporter pSB1075 were diluted to an equalised OD_{600} of 1.0. Molten LB agar was cooled to 40°C, at which point 1 ml of the equalised bioreporter culture was added to each assay plate, with separate plates set up with pSB536 and pSB1075. Once set, holes were cut in the agar with the rear end of a 100 µl pipette tip, and the agar removed. The filtered supernatants were then added to these holes, 90 µl per hole, and the plates incubated at 37°C under static conditions and without inversion for 1 hour. Following the incubation, the light output was assessed using a Hamamatsu Light Camera running Wasabi imaging

software. If the relevant AHL was present, then a halo of light was observed around the hole containing the supernatant.

Statistical Analysis

Statistical analysis of all acquired data was performed using Graphpad Prism 7, (http://www.graphpad.com/scientific-software/prism/).

Genome sequencing

Samples were prepared for sequencing by DNA extraction using a Qiagen DNeasy Blood and Tissue kit. Once extracted, DNA concentrations were recorded using a Qubit Fluorometer and sent for sequencing to Dr Alan McNally at Nottingham Trent University. Once the data was returned, analysis of SNP location and function was performed using Artemis, (http://www.sanger.ac.uk/science/tools/artemis).

Results

Designing a *C. elegans* model serial passage experiment for *P. aeruginosa*

To monitor whether varying host density alters the phenotype and genotype of evolving *P. aeruginosa* populations, a novel *in vivo* experimental protocol was developed. In creating this protocol several factors were required for it to be considered a successful model. Firstly, the *C. elegans* host must be able to survive a *P. aeruginosa* infection long enough to transmit the bacteria horizontally to uninfected adult worms. If *P. aeruginosa* infection was only transmitted vertically in the experimental model, then host density could not be considered an evolutionary factor. This is because the infection would only be occurring during worm reproduction and not between worms in the environment. Secondly, bacterial cells growing externally on *C. elegans* worms would have to be limited. This is because the initial infection would take place on a lawn of *P. aeruginosa*. Therefore, the starting infected population would have substantial amounts of bacteria carried externally. This is not representative of natural bacterial loads, and not the focus of this study, which was examining bacteria evolving inside worms. Finally, a method to alter the rate of transmission was required to test the central hypothesis of this work; that altered host density (and therefore transmission between worms) provides evolutionary pressures to select for altered virulence.

Confirming infection in the serial passage experiment

To establish the optimal conditions for the serial passage experiment, several trial assays were performed, using the method previously outlined. An example of an infected, Pharynx-tagged, worm can be seen in Figure 3.2. This methodology was used to establish that an incubation time of 72 h gave the best rate of infection transmission (Figure 3.3), so this was the incubation time taken forward into the main selection experiment. This set up was repeated using both regular and Pharynx-tagged *C. elegans* as the initial infected population, to ensure that the presence of the fluorescent tag did not affect the result. The time of 72 h is also a time period where high rates of worm death[161] should minimise the chances of individuals from the initially infected group being carried over into later passages of the experiment.

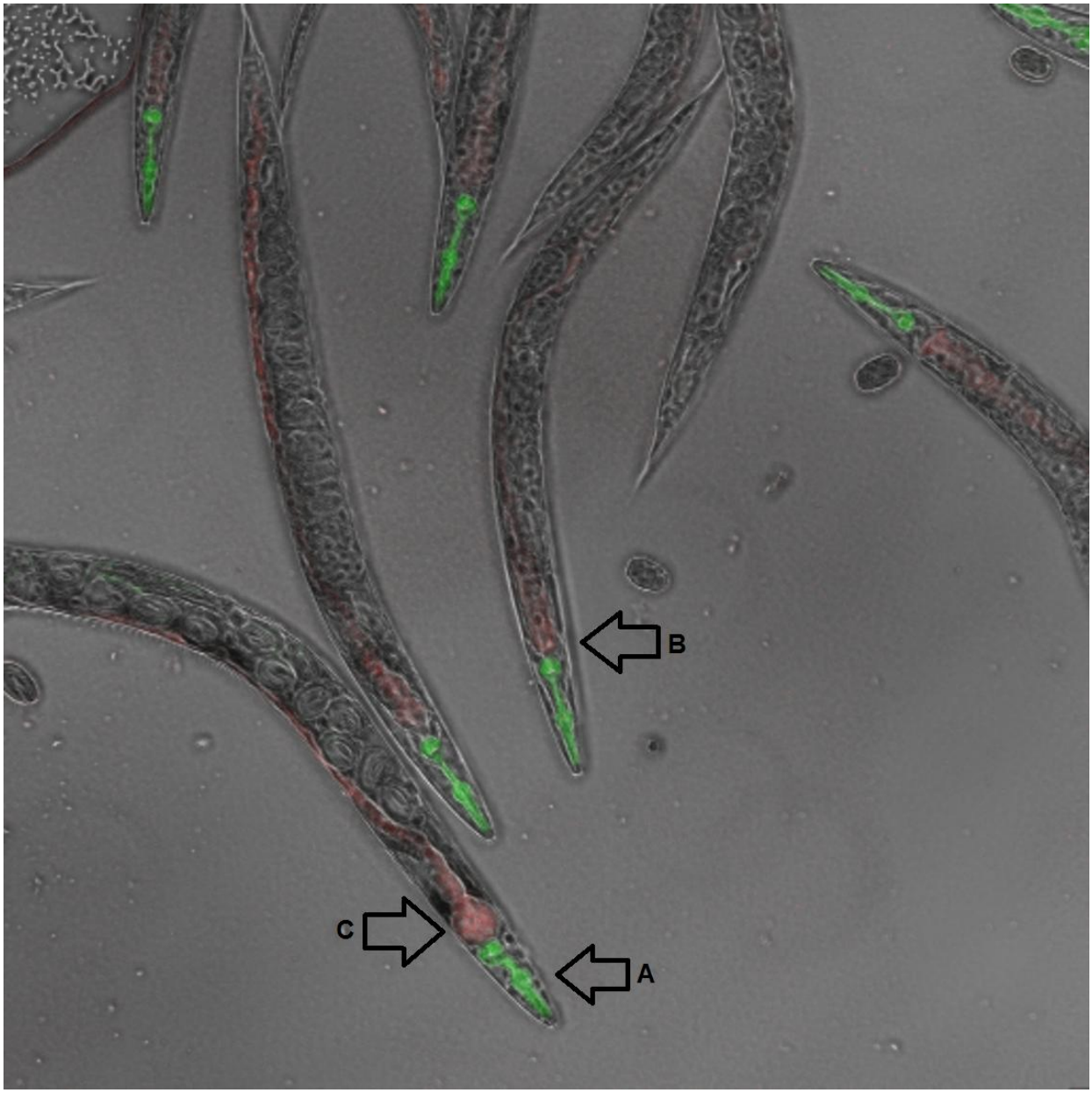


Figure 3.2: Pharynx GFP tagged *C. elegans* with mCherry tagged *P. aeruginosa* present in their digestive systems. The tagged pharynx (A), start of the worm's digestive tract (B) and a high concentration of florescent *P. aeruginosa* (C) are marked. These two fluorescent markers were used to determine whether *P. aeruginosa* could be transmitted between *C. elegans* hosts.

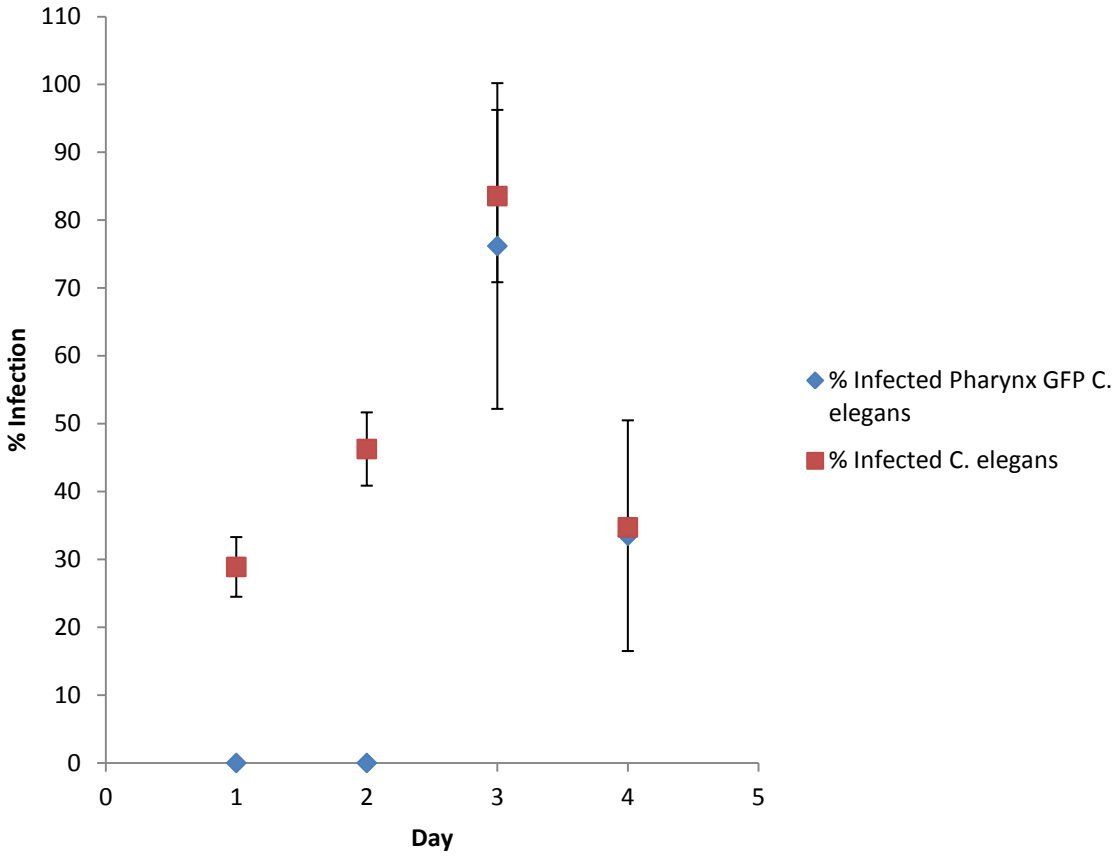


Figure 3.3: Infection rates of *C. elegans* by *P. aeruginosa*. Displayed are the infection rates of the initially uninfected *C. elegans* phenotype for both variations performed, a starting infected population of wildtype plus uninfected Pharynx-tagged, and vice versa. Each data point represents the average of three replicates, with error bars showing standard deviation. Plates were incubated at 22°C. Although each replicate started with 15 uninfected worms, variable numbers were detected on each day of the assay. Despite initial lack of infection amongst the Pharynx GFP population, by the third day the infection rate had caught up with that of the untagged population. The fall in infection on the fourth day was attributed to infected worms beginning to die and the slow kill phenotype fully manifesting. Therefore, this data would suggest that 3 day passage rounds maximise infection levels of *C. elegans* by *P. aeruginosa*.

Transmission occurs at high and low host densities

Different sized plates were used to achieve different worm densities. However, there were concerns that the larger plates, creating a low host density environment, would not have reliable transmission to fresh hosts. To ensure that transmission was possible for both intended host densities, infection percentages were assessed after 72 h, using the same methodology used to confirm infection. Both high host density (54 mm plate diameter) and low host density (168 mm plate diameter) were tested. Substantial proportions of *C. elegans* were infected after 72 h for both host densities (Figure 3.4), and so both were viable for the long-term selection experiment.

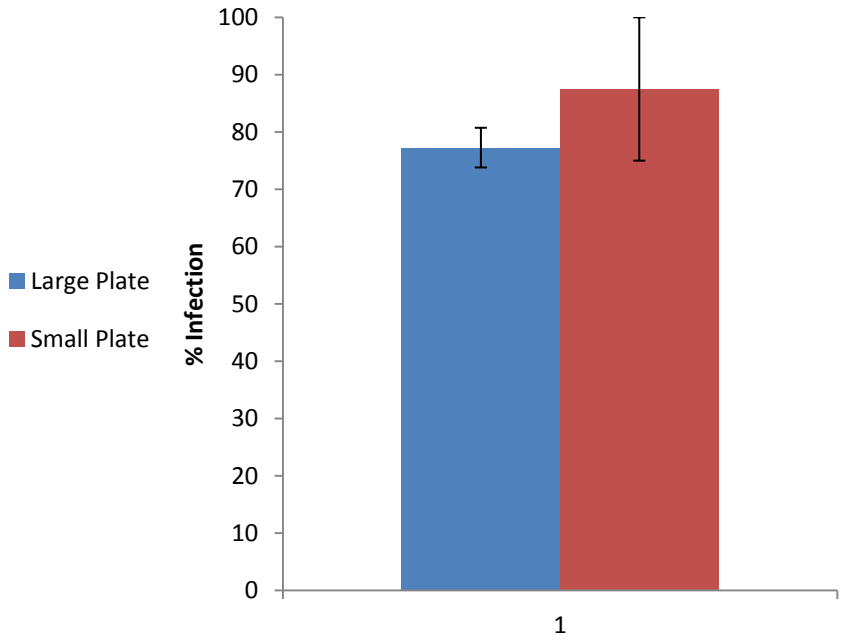


Figure 3.4: Infection levels of *C. elegans* populations at different host density levels. Levels of *P. aeruginosa* infection were assessed after 72 h. Each data set represents the average of a triplicate of samples, with standard deviation shown by error bars. Both host densities have enough infection present that picking worms at random should result in a predominantly infected starting population for the next round of passage.

Antibiotic Wash Reduces External *C. elegans* bacterial load

The initial infection step of the selection method, in which *C. elegans* are infected on a lawn of *P. aeruginosa*, results in large numbers of bacteria adhering to the worms. To reduce this, an antibiotic wash step was included. Of the three conditions used to test the efficacy of the antibiotic wash, the Sm washed population produced the smallest bacterial coverage after incubation for 72 hours (Figure 3.5). Therefore, this wash step had the desired effect in limiting the external bacterial load, and was incorporated into the final protocol for the selection experiment.

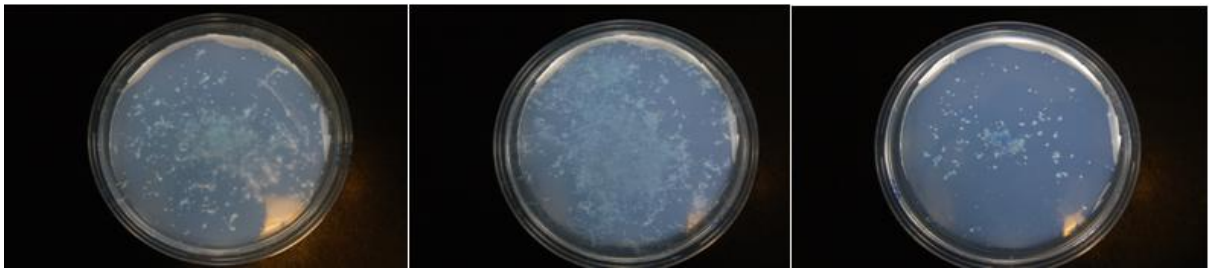


Figure 3.5: Reduction of worm bacterial load by Sm wash, results are representative examples of 3 biological replicate experiments. Shown is the bacterial growth from directly picking *C. elegans* off the *P. aeruginosa* lawn used to establish infection (left), growth when worms were washed from the plate using M9 buffer (middle), and growth after washing off with M9 and a 10 minute wash with Sm (200 µg ml^{-1}). All plates initially had 20 individual *C. elegans* added and were incubated at 22°C for 72 hours before imaging. The Sm wash resulted in far lower bacterial growth, which suggested that it had successfully washed off the artificially large quantity of external bacteria the *C. elegans* would have acquired on the infection plate.

Some evolved populations did not persist through all rounds of passage

Although the designed protocol was ultimately successful in generating evolved *P. aeruginosa* populations, some evolving lines failed to be transmitted at certain points throughout the experiment. Therefore, of the six initial replicate lines for each host density condition, only four persisted for the full 20 rounds of selection, resulting in 20 evolved populations in total. Four each from the low and high host density treatment groups, and six from each of the no worm control groups.

High host density evolved populations retain virulence towards *C. elegans*

Most of the low host density populations lost the *C. elegans* killing phenotype, with worm populations rapidly becoming uncountable, despite the presence of potentially pathogenic bacteria (Figure 3.6A). Only the L4 population retained any level of virulence, only being slightly attenuated vs. the ancestor. All other populations diverged by a statistically significant amount (Dunnett's multiple comparison test, p values <0.0001). The control populations, while somewhat similar, did display more variation in virulence (Figure 3.6B). Although the majority of control populations did display an avirulent phenotype, 2 populations, L1C and L4C, did retain virulence levels close to the ancestor strain, and similar to the worm evolved L4 population. Both of these populations, along with the fully avirulent ones, were significantly different from the ancestor (Dunnett's multiple comparison test, p values <0.007). However, the quantitative difference between both L1C and L4C and the ancestor was small enough that both evolved populations should still be considered virulent towards *C. elegans*.

So overall, there was little real difference between the worm evolved and control treatment groups.

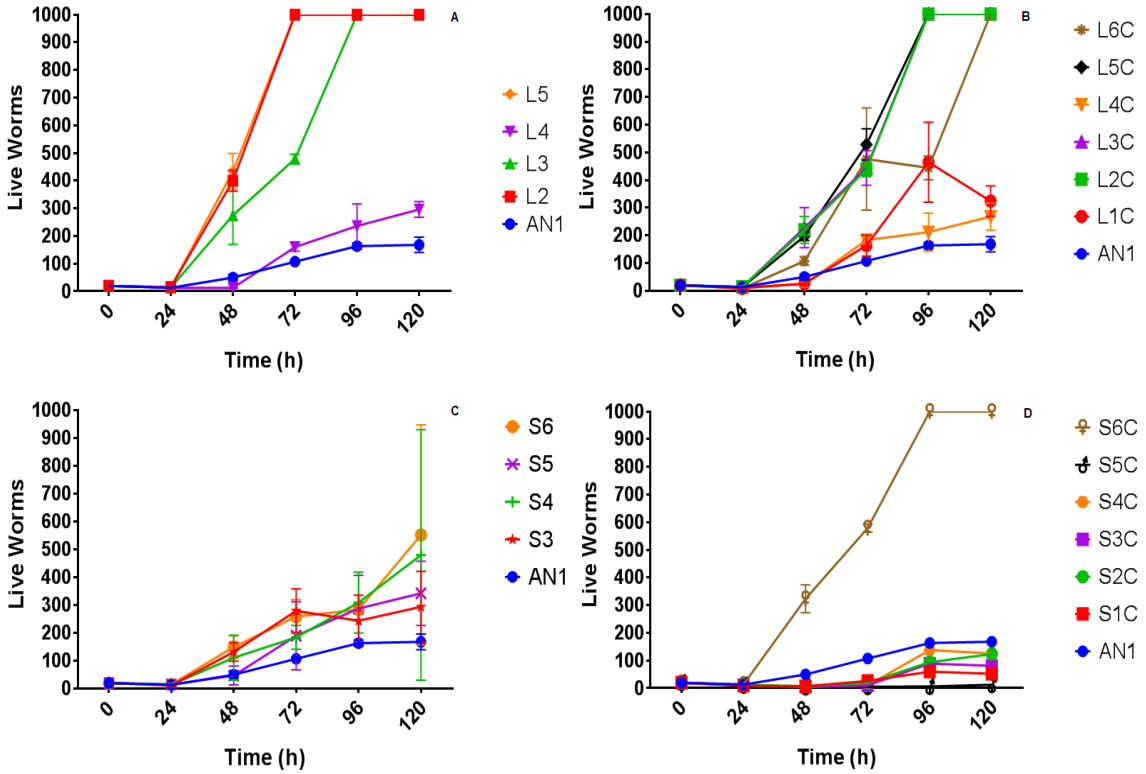


Figure 3.6: Worm killing by evolved *P. aeruginosa* populations. AN1 is the Ancestor strain, L denotes low host density worm evolved conditions, LC are low host density controls, S are high host density worm evolved and SC are high host density controls. All results are shown as means, three replicates per treatment. When error bars aren't visible, the bars are smaller than the symbols. The majority of low host density worm evolved (A) and low host density control (B) populations lost virulence, with only 1 worm evolved and 2 control populations retaining worm killing ability. The high host density treatment groups retained virulence more often, with no worm evolved populations (C) and only 1 control (D) losing virulence. Note, the large error bars for the S4 and S6 populations are due to one of the 3 replicates being substantially larger than the other 2.

The high host density populations showed more consistency than the low density populations. Although the worm evolved populations did display some variation, with substantially larger live worm populations than was seen with the ancestor strain (especially S4 and S6), all populations retained a level of virulence so that *C. elegans* numbers did not reach uncountable densities (Figure 3.6C). The majority of populations showed statistically significant differences compared to the ancestor (Dunnett's multiple comparison test, p values <0.01), although all retained some virulence towards *C. elegans*. The only exception was S5 (p = 0.678), however the raw data for this population was consistent with the trend seen in the rest of its treatment group, retention of virulence. The results for the high host density controls were mostly similar to the worm evolved (Figure 3.6D). Most of the populations retained virulence levels comparable to that of the ancestor, they demonstrated less virulence variation than the worm evolved lines, with fewer live worms present. One population, S5C, demonstrated a small but significant increase in virulence (Dunnett's multiple comparison test, p values <0.02). The one exception to this trend was the S6C population, which had reduced in virulence (Dunnett's multiple comparison test, p values <0.0001).

Worm killing by individual isolates taken from populations

Low host density isolates displayed levels of virulence comparable to the virulence of the population from which they were taken (Figure 3.7). The only set of isolates which fully retained virulence were those taken from the L4 population (Figure 3.7C). The other three population groups demonstrated attenuated virulence, with worm populations reaching uncountable levels (Dunnett's multiple comparison test, p values <0.0001). Isolates taken from the control populations demonstrated more variation in virulence. Most of the population groups contained examples of both attenuated and preserved virulence towards *C. elegans* (Figure 3.8). The 2 exceptions to this were L2C and L5C, which were 100% attenuated. For the four mixed populations loss of virulence was still somewhat favoured, with 2 populations, L3C and L4C having more attenuated virulence isolates than retained, while only a single population, L6C, had more isolates in which virulence was preserved. The last control population, L1C, was an even split, with three isolates following each observed phenotype.

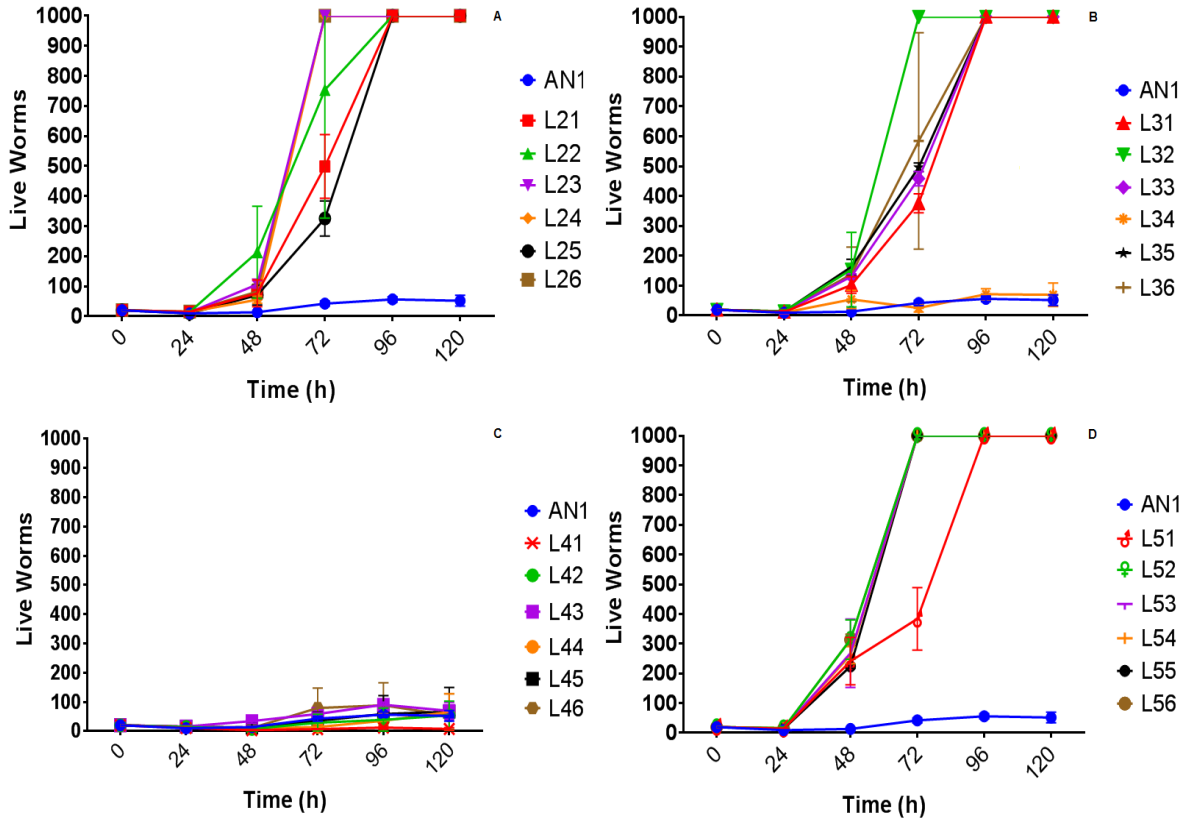


Figure 3.7: Worm killing by low host density, worm evolved *P. aeruginosa* isolates. All results are shown as means, three replicates per treatment. When error bars aren't visible, the bars are smaller than the symbols. Each individual graph shows the isolates taken from a single evolved population, L2 (A), L3 (B), L4 (C) and L5 (D). The vast majority of isolates were avirulent to the *C. elegans*, with only 1 of the L3 isolates, L34, and all the isolates from the L4 population, retaining a virulent phenotype.

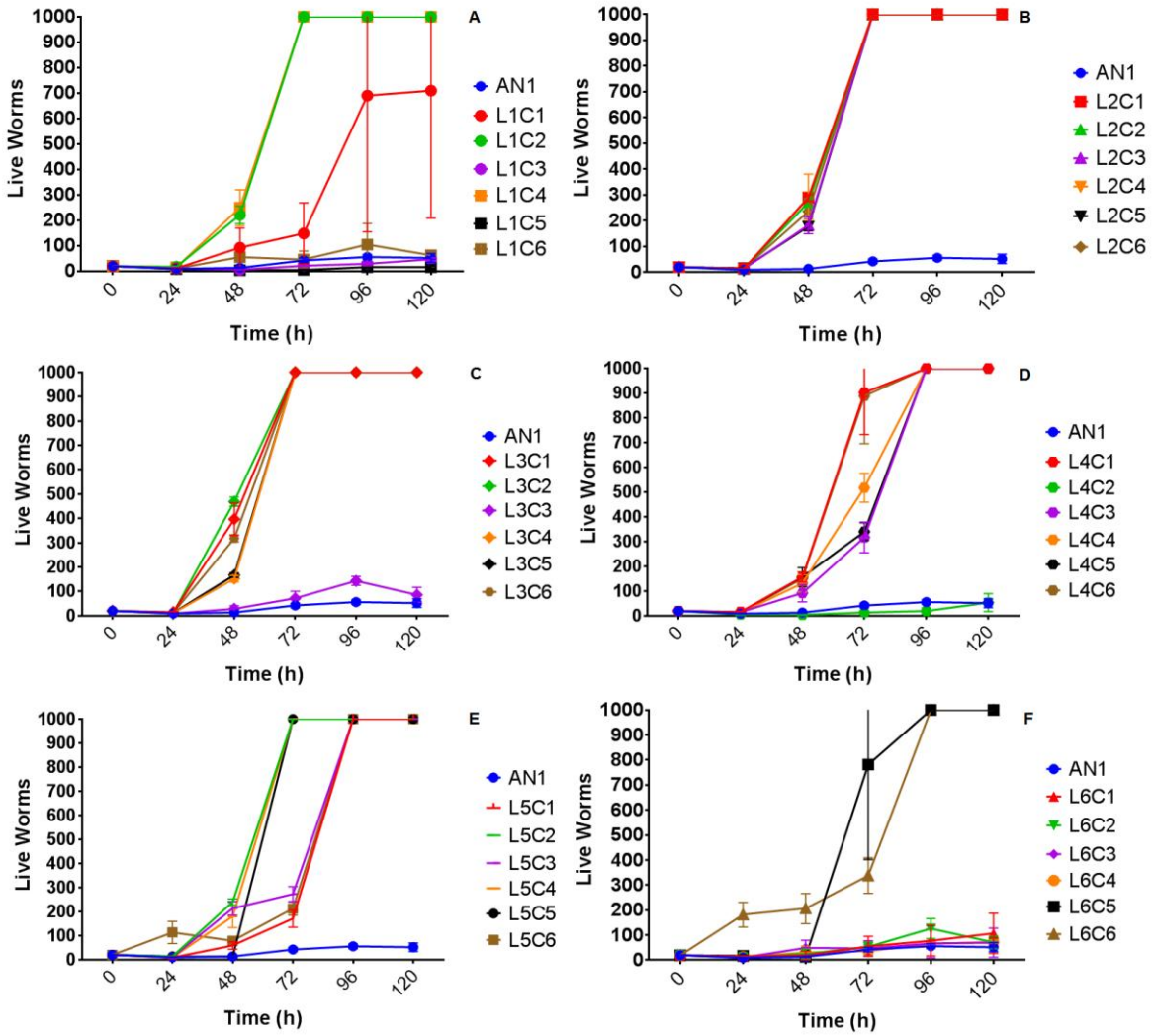


Figure 3.8: Worm killing by low host density control *P. aeruginosa* isolates. All results are shown as means, three replicates per treatment. When error bars aren't visible, the bars are smaller than the symbols. Each individual graph shows the isolates taken from a single evolved population, L1C (A), L2C (B), L3C (C), L4C (D), L5C (E) and L6C (F). The very large error bars observed for L1C1 occurred due to one replicate being substantially smaller than the other 2. Both virulent and avirulent phenotypes were expressed by these isolates, with the majority of population groups containing examples of both phenotypes. However, 2 isolate groups, L2C and L5C, were entirely avirulent.

The high host density isolates mostly retained their virulence in *C. elegans* (Figure 3.9). All isolates which reached uncountable numbers of worms were statistically significant (Dunnett's multiple comparison test, p values <0.0001). In addition, some isolates (S52, S56, S62, S65 and S66), were also significantly different from the ancestor (Dunnett's multiple comparison test, p values <0.0001). However, these isolates retained virulence close to the ancestor levels. The S6 population isolates were the only ones with a homogenous 100% virulent phenotype. The remaining three populations all produced more virulent isolates than avirulent, with only 5 isolates between them with attenuated virulence. The low host density controls followed a similar pattern favouring virulence retention (Figure 3.10). Just like the worm evolved isolates, all the samples which reached uncountable numbers were significantly different from the ancestor (Dunnett's multiple comparison test, p values <0.0001), and a number of additional isolates, S1C3, S2C1, S2C2, S3C1, S3C5, S3C6, S4C3, S4C5 and S5C4 had a statistical difference, though the actual virulence attenuation was only small, and so were still considered to be virulent. While only one population group, S1C, retained the virulent phenotype in 100% of its isolates, three others did produce more virulent isolates than attenuated. Even the two populations that did favour attenuated virulence overall, S4C and S6C, were not homogenous, still possessing some isolates which retained their virulence.

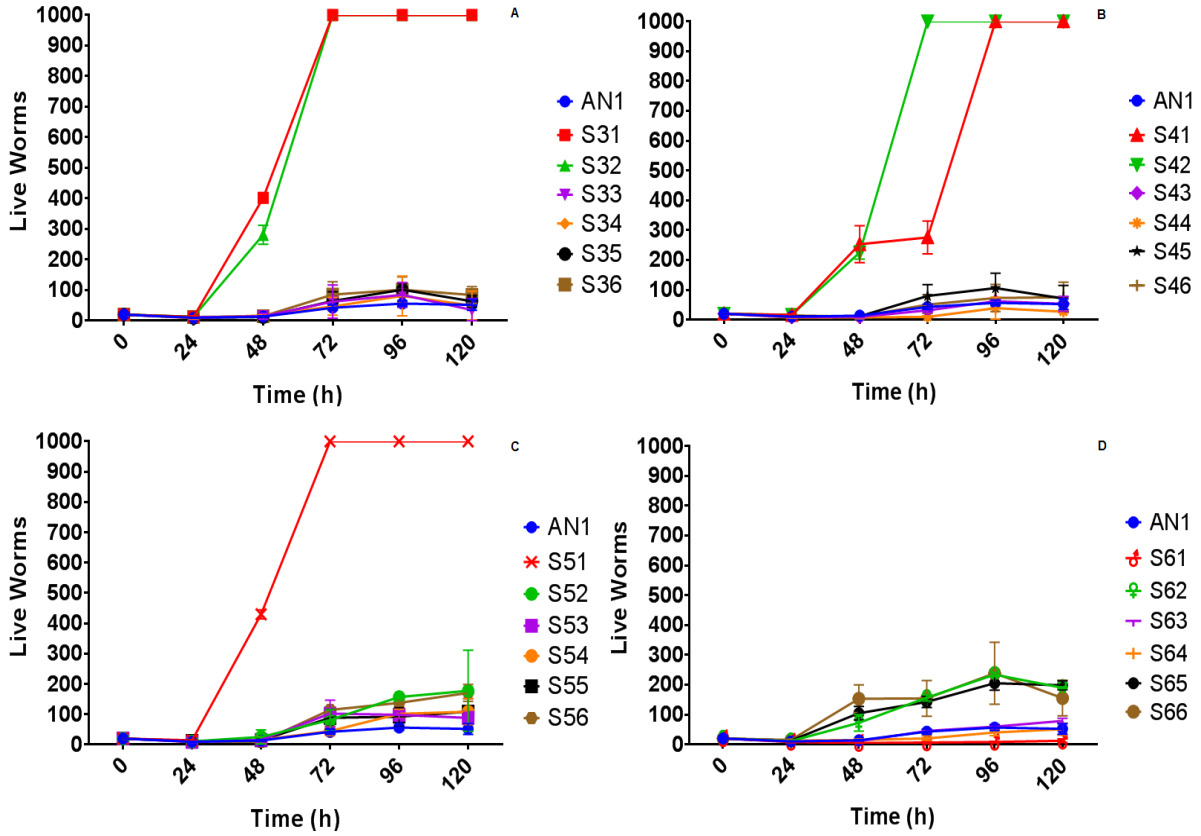


Figure 3.9: Worm killing by high host density, worm evolved *P. aeruginosa* isolates. All results are shown as means, three replicates per treatment. When error bars aren't visible, the bars are smaller than the symbols. Each individual graph shows the isolates taken from a single evolved population, S3 (A), S4 (B), S5 (C) and S6 (D). Although only 1 group of isolates (S6) did not have a mix of phenotypes present, the majority of isolates retained their virulence towards *C. elegans*, with only 5 of the 24 isolates in this cohort developing the avirulent phenotype.

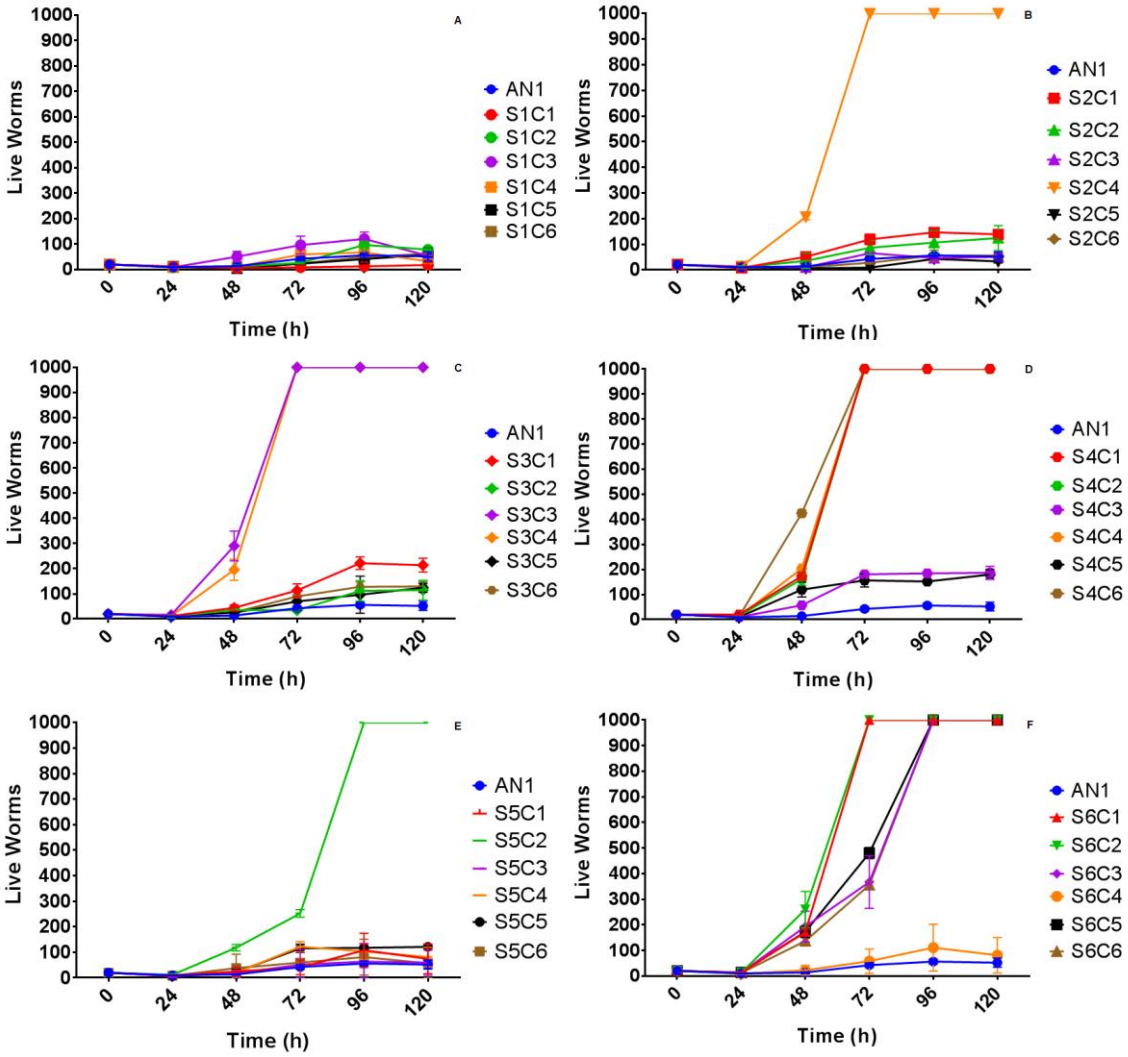


Figure 3.10: Worm killing by high host density control *P. aeruginosa* isolates. All results are shown as means, three replicates per treatment. When error bars aren't visible, the bars are smaller than the symbols. Each individual graph shows the isolates taken from a single evolved population, S1C (A), S2C (B), S3C (C), S4C (D), S5C (E) and S6C (F). Although only the S1C isolate group had 100% virulence retention, the rest of the isolate groups were still predominantly virulent to *C. elegans*, as only 2 other isolate groups, S4C and S6C, had more avirulent isolates than virulent.

Specific combinations of virulence factors are required for *P. aeruginosa* virulence in *C. elegans*

Bacteria	Protease	Cyanide	C4-HSL	3-oxo-C12-HSL	Pyocyanin	Pyoverdin	Pyochelin	*lasB*	Worm Virulence
AN1	+	+	+	+	+	+	+	+	+
Populations									
L2	-	-	+	-	-	+	R	-	-
L3	-	-	+	-	-	R	R	-	-
L4	+	+	+	+	+	R	R	-	+
L5	-	-	-	-	-	R	R	-	-
L1C	+	+	+	+	+	R	R	+	+
L2C	-	-	-	-	-	R	R	-	-
L3C	-	-	+	-	-	R	R	+	-
L4C	+	+	+	+	+	R	R	+	+
L5C	-	-	+	+	-	R	R	+	-
L6C	-	-	+	+	-	R	R	+	-
S3	+	+	+	+	+	R	R	-	+
S4	+	+	+	-	+	R	R	-	+
S5	+	+	+	+	+	R	R	-	+
S6	+	+	+	+	+	R	R	-	+
S1C	+	+	+	+	+	R	R	+	+
S2C	+	+	+	+	+	R	R	+	+
S3C	+	+	+	+	+	R	R	+	+
S4C	+	+	+	+	+	R	R	+	+
S5C	+	+	+	+	+	R	R	+	+
S6C	-	-	-	-	-	R	R	-	-
Individuals									
L21	-	-	-	-	-	R	+	-	-
L22	-	-	-	-	-	+	+	-	-
L23	-	-	-	-	-	+	+	-	-
L24	-	-	-	-	-	+	+	-	-
L25	-	-	-	-	-	+	+	-	-
L26	-	-	-	-	-	+	+	-	-
L31	-	-	-	-	-	R	R	-	-
L32	-	-	-	-	-	+	+	-	-
L33	-	-	-	-	-	+	+	-	-
L34	-	+	+	+	+	R	R	-	-
L35	-	-	-	-	-	+	+	-	-
L36	-	-	-	-	-	+	+	-	-
L41	+	+	+	+	+	R	R	-	+
L42	+	+	+	+	+	+	+	-	+
L43	+	+	+	+	+	R	R	-	+
L44	+	+	+	+	+	R	R	-	+
L45	+	+	+	+	+	+	+	-	+
L46	+	+	+	+	+	+	+	-	+
L51	-	-	-	-	-	+	+	-	-
L52	-	-	-	-	-	+	+	-	-
L53	-	-	-	-	-	+	+	-	-

Bacteria	Protease	Cyanide	C4-HSL	3-oxo-C12-HSL	Pyocyanin	Pyoverdin	Pyochelin	*lasB*	Worm Virulence
L54	-	-	-	-	-	+	+	-	-
L55	-	-	-	-	+	+	+	-	-
L56	-	-	-	-	-	+	+	-	-
L1C1	-	+	-	-	+	R	R	-	-
L1C2	-	-	-	-	-	+	+	-	-
L1C3	+	+	+	+	+	+	+	-	+
L1C4	-	-	-	-	-	+	+	-	-
L1C5	+	+	+	+	+	R	R	-	+
L1C6	+	+	-	+	+	+	+	-	+
L2C1	-	-	-	-	-	R	R	-	-
L2C2	-	-	+	+	+	+	+	-	-
L2C3	-	-	+	+	+	+	+	-	-
L2C4	-	-	-	-	-	R	R	-	-
L2C5	-	-	-	-	-	+	+	-	-
L2C6	-	-	-	-	-	+	+	-	-
L3C1	-	-	-	-	-	R	R	-	-
L3C2	-	-	-	-	-	+	R	-	-
L3C3	+	+	+	+	+	R	R	+	+
L3C4	-	-	-	-	-	R	R	-	-
L3C5	-	-	-	-	-	+	+	-	-
L3C6	-	-	-	-	-	+	+	-	-
L4C1	-	-	-	-	-	+	+	-	-
L4C2	+	+	+	+	+	+	+	+	+
L4C3	+	+	+	-	-	+	+	+	-
L4C4	-	-	-	-	-	+	+	-	-
L4C5	+	+	+	+	-	+	+	+	-
L4C6	-	-	-	-	-	+	+	-	-
L5C1	-	+	+	+	-	R	R	+	-
L5C2	-	-	-	-	-	+	+	-	-
L5C3	-	+	+	+	-	+	+	+	-
L5C4	+	-	-	-	-	+	+	-	-
L5C5	+	+	-	-	-	+	+	-	-
L5C6	+	+	-	-	-	+	+	-	-
L6C1	+	+	+	+	+	+	R	+	+
L6C2	+	+	+	+	+	+	R	+	+
L6C3	+	+	-	+	+	+	R	+	+
L6C4	+	+	+	+	+	+	+	+	+
L6C5	-	-	-	-	-	+	+	-	-
L6C6	-	-	-	-	-	+	R	-	-
S31	-	-	-	-	-	+	+	-	-
S32	-	-	-	-	-	+	+	-	-
S33	+	+	+	+	+	R	R	-	+
S34	+	+	+	+	+	+	+	-	+
S35	+	+	+	+	+	+	+	-	+
S36	+	+	+	+	+	+	+	-	+
S41	-	+	+	+	-	+	+	-	-
S42	-	-	-	-	-	+	+	-	-
S43	+	+	+	+	+	+	+	-	+

Bacteria	Protease	Cyanide	C4-HSL	3-oxo-C12-HSL	Pyocyanin	Pyoverdin	Pyochelin	*lasB*	Worm Virulence
S44	+	+	+	+	+	+	+	-	+
S45	+	+	+	+	+	+	+	-	+
S46	+	+	+	+	+	R	+	-	+
S51	-	-	-	-	-	+	+	-	-
S52	+	+	+	+	+	+	+	-	+
S53	+	+	+	+	+	R	R	-	+
S54	+	+	+	+	+	+	+	-	+
S55	+	+	+	+	+	+	+	-	+
S56	-	+	+	+	+	+	+	-	+
S61	-	+	+	+	+	+	+	-	+
S62	+	+	+	+	+	R	+	-	+
S63	+	+	+	+	+	+	+	-	+
S64	+	+	+	+	+	R	R	-	+
S65	+	+	+	+	+	+	+	-	+
S66	+	+	+	+	+	R	R	-	+
S1C1	+	+	+	+	+	+	+	+	+
S1C2	+	+	+	+	+	R	+	+	+
S1C3	+	+	+	+	+	+	+	-	+
S1C4	+	+	-	+	+	R	R	-	+
S1C5	+	+	+	+	+	R	R	+	+
S1C6	+	+	+	+	+	R	+	+	+
S2C1	+	+	+	+	+	R	+	-	+
S2C2	+	+	+	+	+	R	R	+	+
S2C3	+	+	+	+	+	R	R	+	+
S2C4	+	-	-	+	-	+	+	-	-
S2C5	+	+	+	+	+	R	R	+	+
S2C6	+	+	+	+	+	+	+	-	+
S3C1	+	+	+	+	+	+	+	+	+
S3C2	+	+	+	+	+	+	+	+	+
S3C3	-	-	-	-	-	+	+	+	-
S3C4	-	-	-	-	-	+	+	+	-
S3C5	+	+	+	+	+	R	R	+	+
S3C6	+	-	+	+	+	+	+	+	+
S4C1	-	-	-	-	-	+	+	-	-
S4C2	-	+	+	+	-	+	+	-	-
S4C3	+	+	+	+	+	+	+	-	+
S4C4	-	-	-	-	-	R	R	+	-
S4C5	+	+	+	+	+	+	+	-	+
S4C6	-	-	-	-	-	+	+	-	-
S5C1	+	+	+	+	+	R	R	-	+
S5C2	-	+	+	+	-	+	+	-	-
S5C3	+	+	-	-	+	+	+	-	+
S5C4	+	+	+	+	+	-	R	-	+
S5C5	+	+	+	+	+	+	+	-	+
S5C6	+	+	+	+	+	+	+	-	+
S6C1	-	-	-	-	-	+	+	-	-
S6C2	-	-	-	-	-	R	R	-	-
S6C3	-	-	-	-	-	+	+	-	-

Bacteria	Protease	Cyanide	C4-HSL	3-oxo-C12-HSL	Pyocyanin	Pyoverdin	Pyochelin	*lasB*	Worm Virulence
S6C4	+	+	-	+	+	+	+	-	+
S6C5	-	-	-	-	-	+	+	-	-
S6C6	-	-	-	-	-	+	+	-	-

Table 3.2: Summary of *P. aeruginosa* virulence factor phenotype assays. Note that for the siderophore results most were either at the level of the ancestor, and were marked as '+' or presented reduced expression, but some was retained, marked as 'R'.

In addition to the virulence assays previously described, the evolved *P. aeruginosa* populations and isolates were tested for an array of previously described virulence factors, as well as the QS signal molecules C4-HSL and 3-oxo-C12-HSL (Table 3.2).

At least some siderophore activity was retained in most of the tested populations and individual isolates (Table 3.2, Figure 3.11). This was the case for both pyoverdin and pyochelin, with no completely negative results for pyochelin, and only one (S5C4) for pyoverdin. However, siderophore levels did not correlate with overall virulence (Table 3.3), as both virulent and avirulent *P. aeruginosa* samples were found for all siderophore phenotypes, such as L22, L41, L42 and S6C2 (Table 3.2).

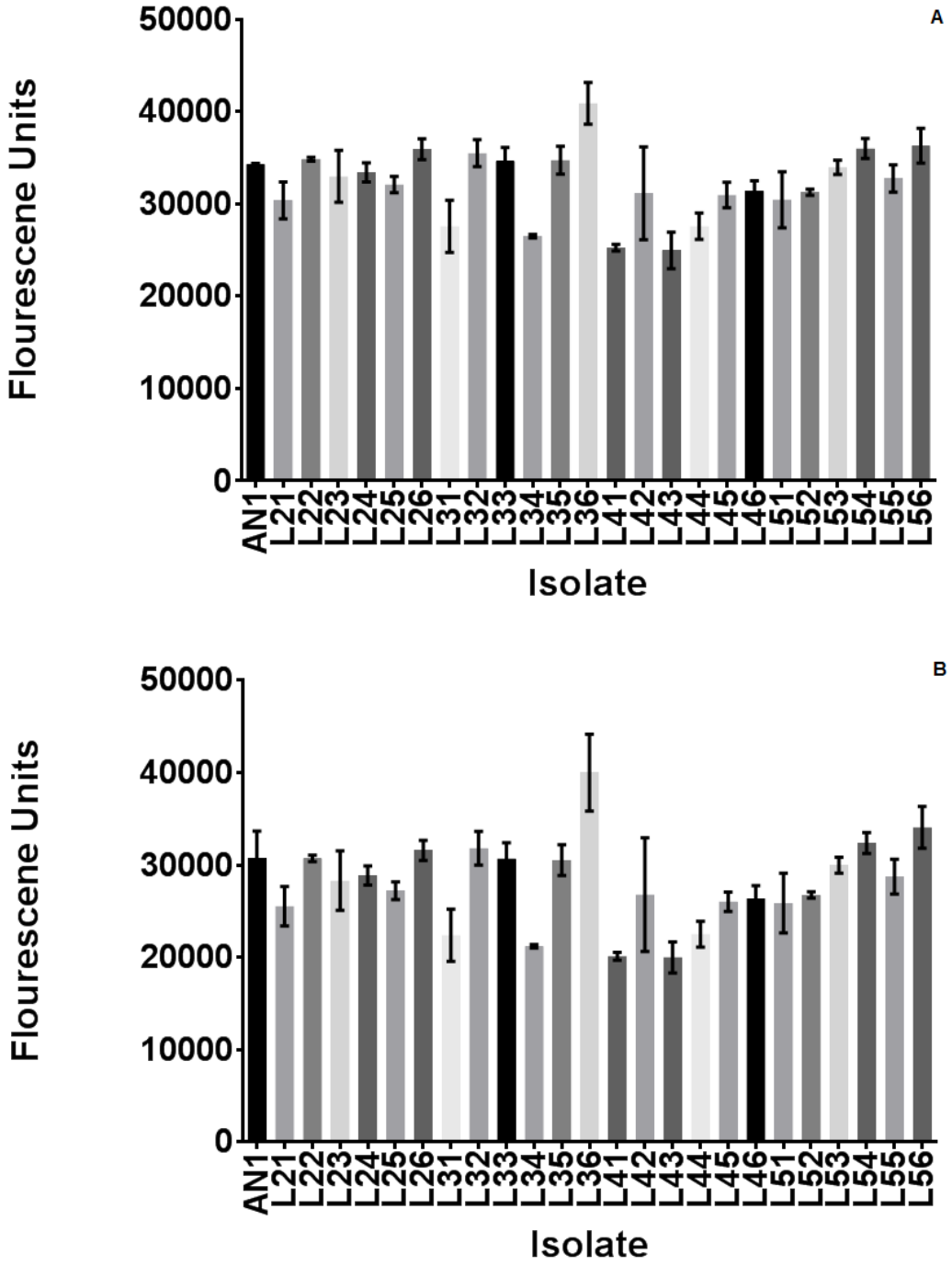


Figure 3.11: Examples of siderophore phenotypes. Displayed are the pyoverdin (A) and pyochelin (B) phenotypes of the low host density worm evolved isolates of *P. aeruginosa*. Although a variety of levels of siderophore production were observed, with examples of both higher (L36) and lower (L34) production than the AN1 ancestor, some siderophore production was still retained. Full data sets of all tested populations and isolates can be seen in Appendix 1.

	C4-HSL	3-oxo-C12-HSL	Pyocyanin	Protease	Cyanide	Pyoverdin	Pyochelin	*lasB*	Virulence
C4-HSL	**NA**	0.87	0.73	0.66	0.769	-0.282	-0.222	0.325	0.724
3-oxo-C12-HSL	0.87	**NA**	0.831	0.761	0.841	-0.242	-0.182	0.318	0.826
Pyocyanin	0.73	0.831	**NA**	0.815	0.816	-0.246	-0.187	0.292	0.931
Protease	0.66	0.761	0.815	**NA**	0.832	-0.197	-0.139	0.299	0.888
Cyanide	0.769	0.841	0.816	0.832	**NA**	-0.222	-0.163	0.222	0.838
Pyoverdin	0.014	0.035	0.167	-0.038	-0.01	**NA**	0.817	-0.269	0.107
Pyochelin	-0.222	-0.182	-0.187	0.139	0.163	0.817	**NA**	-0.304	-0.82
lasB	0.325	0.318	0.292	0.299	0.222	-0.269	-0.304	**NA**	0.329
Virulence	0.724	0.826	0.931	0.888	0.838	-0.241	-0.182	0.329	**NA**

Table 3.3: Correlation of virulence factors and *C. elegans* virulence across all tested samples of *P. aeruginosa* (Pearson correlation coefficient). Virulence was most correlated with five virulence factors, C4-HSL, 3-oxo-C12-HSL, Protease, Pyocyanin and Cyanide (r values above 0.7).

At the population level, *lasB* (codes for elastase production) expression was lost in all the worm evolved populations, while the majority of the control populations retained activity (Table 3.2). Only the L2C and S6C controls lost *lasB* expression at the population level. Individual isolates taken from worm evolved populations were universally negative, just like the population level data. Isolates from control populations were more varied, with both *lasB* positive and negative isolates found in most population groups. Four groups were exceptions here, with the L1C, L2C, S5C and S6C population groups all remaining 100% *lasB* negative, despite two of those being positive at the population level. Ultimately however, this promoter activity does not appear to exert any influence over virulence in *C. elegans*, as there was little correlation between worm killing and *lasB* promoter activity (Table 3.3).

For pyocyanin, exogenous protease and cyanide, the phenotypes were very similar at the population level (Table 3.2). The majority of low host density populations, regardless of whether they were worm evolved or control, showed a loss of production of these virulence factors, while they were retained in the two high host density treatment conditions. This was not the case for both C4-HSL and 3-oxo-C12-HSL signal molecules (Table 3.2). Although a number of populations did lose 3-oxo-C12-HSL (L2, L3, L3C and S4), and several more lost production of both AHLs (L5, L2C, S6C), the overall trend was for AHL retention. 85% of populations retained C4-HSL and 65% retained 3-oxo-C12-HSL.

The presence of AHLs had no effect on siderophore production, with examples present of siderophores being both attenuated and retained in both the

presence and absence of the 2 AHLs (Table 3.2), as well as an overall small negative correlation between both AHLs and both siderophores (Table 3.3). This suggests that QS does not control the synthesis of the siderophore molecules. However, the presence of 3-oxo-C12-HSL correlated with the trio of virulence factors pyocyanin, exogenous protease and cyanide in the majority of populations (Table 3.2, Table 3.3, Figure 3.13). This correlation was not universal. L5C and L6C lost all three virulence factors while retaining the 3-oxo-C12-HSL, while S4 was the opposite, retaining the virulence factors despite a lack of 3-oxo-C12-HSL. This would suggest the situation is more complicated than a simple relationship between 3-oxo-C12-HSL and QS-dependent virulence factors, with other regulatory systems at work as well, rather than a single 'master controller'.

When the individual isolates were examined, the trends for pyocyanin loss were similar to those at the population level, although with more variation (Table 3.2, Figure 3.12). While the majority of low host density isolates, both worm evolved and control, were pyocyanin negative, the opposite was true for the high host density isolates, which mostly retained pyocyanin. However, there were a number of isolates which bucked this trend, around a third of isolates for 3 of the 4 treatment groups, with the high host density worm evolved condition being more homogenous with only a fifth of isolates being pyocyanin negative. It was a similar result for exogenous protease, cyanide and the AHLs, with the vast majority of both low host density treatment conditions being negative for these virulence factors, while the high host density isolates were predominantly positive.

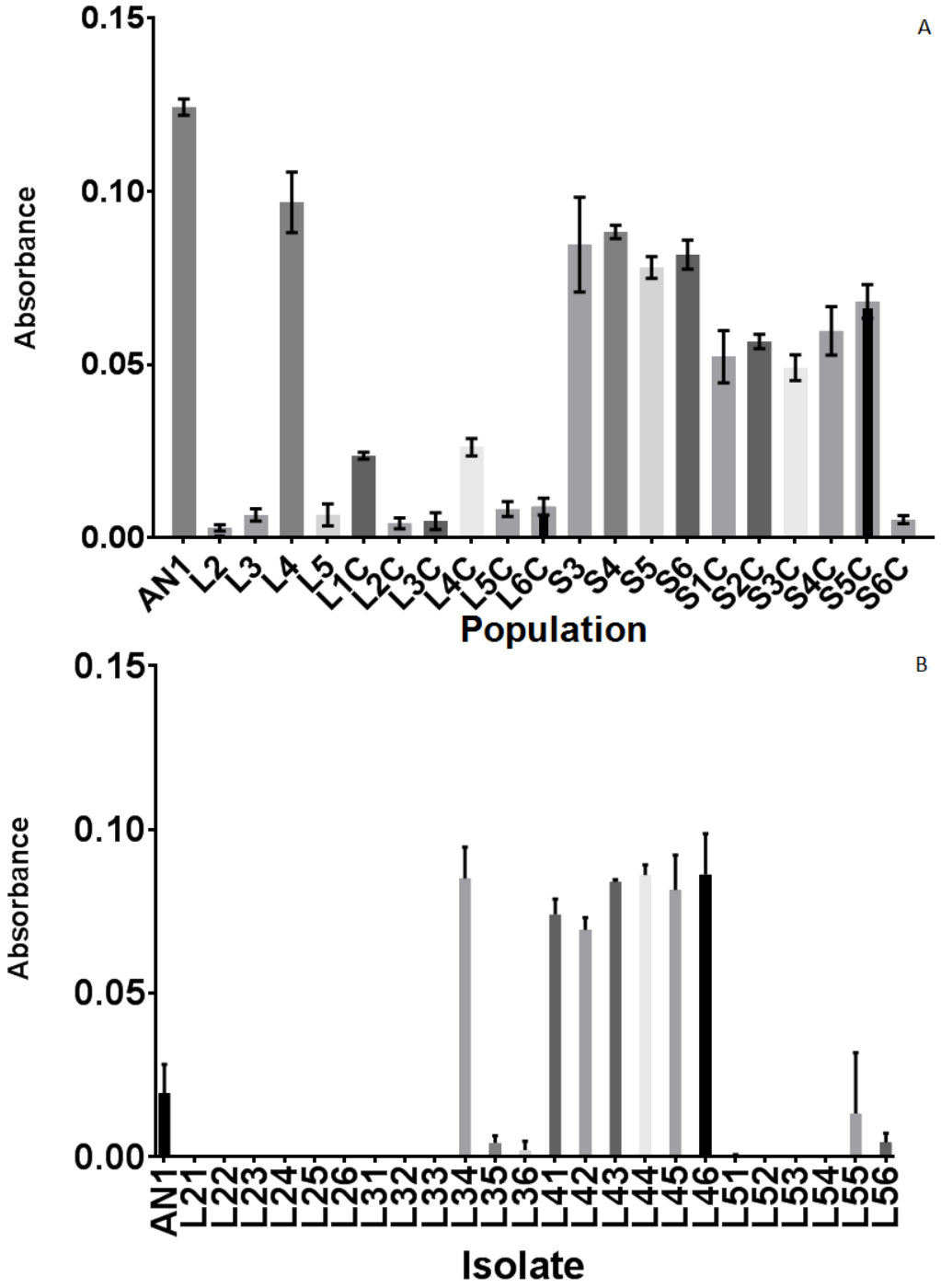


Figure 3.12: Example of the general trend of pyocyanin retention and loss being consistent between *P. aeruginosa* evolved populations (A) and low host density evolved isolates (B). The majority of isolates matched their population of origin, with isolates from L2, L3 and L5 losing pyocyanin, while it was retained in L4. However, some isolates, such as L34 did provide variation, displaying a phenotype notably different to that seen at the population level. Full data sets of all tested populations and isolates can be seen in Appendix 1.

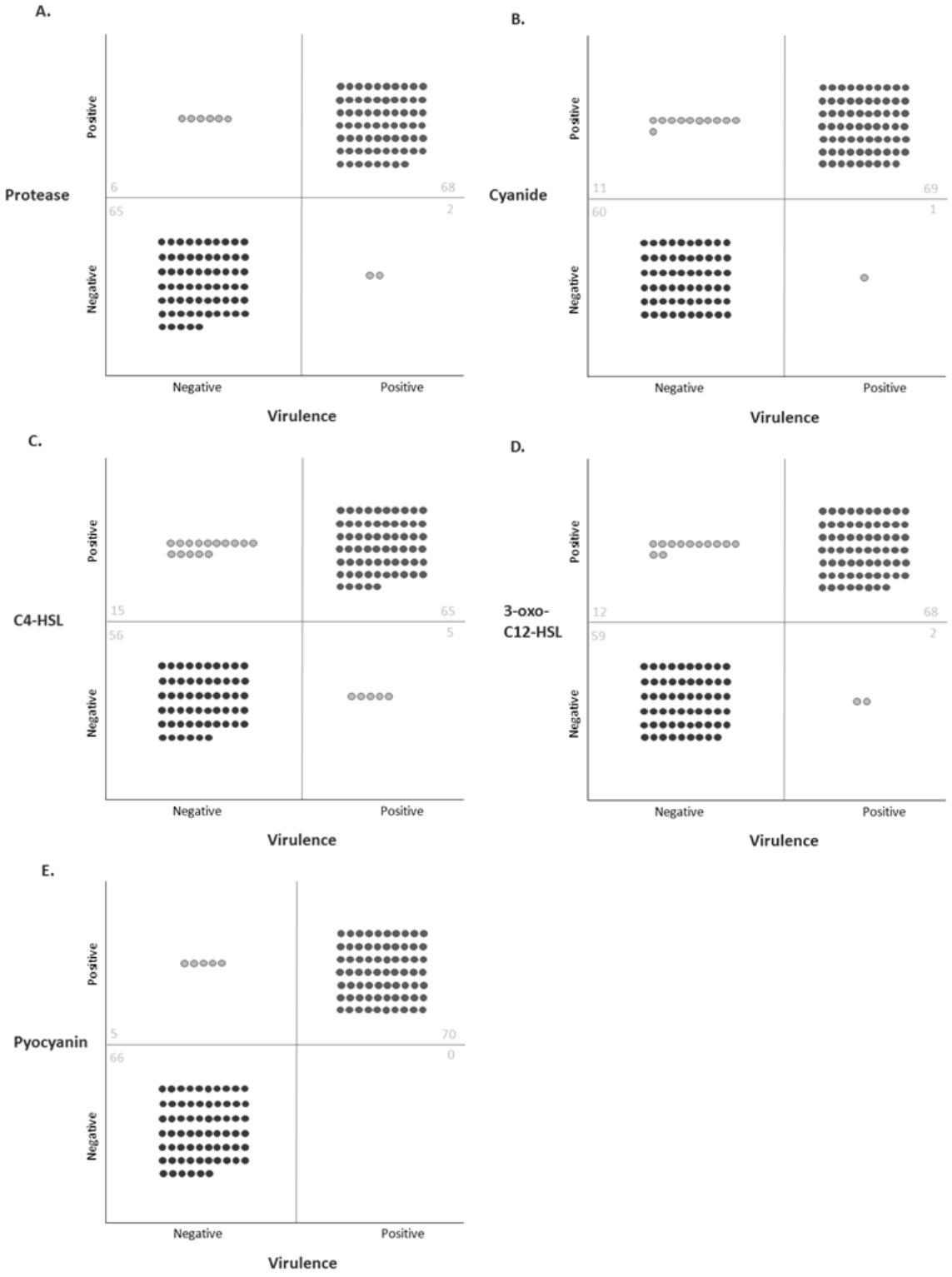


Figure 3.13: Illustrations of the correlations between virulence towards *C. elegans* and tested *P. aeruginosa* virulence factors Protease (A), Cyanide (B), C4-HSL (C), 3-oxo-C12-HSL (D) and Pyocyanin (E) for all 141 *P. aeruginosa* populations and isolates tested (Table 3.2). Most tested *P. aeruginosa* displayed positive or negative phenotypes for each virulence factor and virulence, however the correlations were no absolute, with some mixed positive and negative phenotypes present for each virulence factor.

This array of virulence factors also correlated reasonably closely with worm virulence (Table 3.3, Figure 3.13). The majority of virulent populations and isolates all preserved this battery of virulence factors with 60 of the 69 virulent bacteria also displaying positive phenotypes for all 5 virulence factors (Table 3.2). The minority that did lose some virulence factors while retaining overall virulence preserved activity for most of the five. S61 lacked protease, L1C6, S6C4 and S1C4 lacked C4-HSL, S5C3 lacked both (Table 3.2). Although the presence of cyanide in all worm killing phenotypes would suggest that cyanide was the most important virulence factor controlling killing in *C. elegans*, there were also cyanide positive isolates which were negative for worm killing, including L34. S41, L2C2, L4C3, L5C5, L5C6, S4C2 and S5C2 (Table 3.2, Figure 3.13). The combination of cyanide, pyocyanin and C4-HSL was quite closely correlated with worm virulence, being retained in the majority of worm killing strains, but there were exceptions here too, both of retaining the virulence factors but losing virulence (L34) and losing at least one of the three, but retaining virulence (L1C6, S3C6) (Table 3.2, Figure 3.13). So, while all five of these virulence factors appear to have an important role in virulence towards *C. elegans*, virulence is the result of several of them working together. No single virulence factor is an essential component of the virulence phenotype.

Genomic analysis of evolved populations

Whole genome sequencing of all 20 evolved populations revealed the breadth of SNPs that resulted from each treatment group, with the hope of being able to identify a genomic basis for the previously observed phenotype patterns. The low host density worm evolved treatment resulted in the most SNPs (Figure 3.14). Whilst the L4 population had a low number of SNPs (121), the other evolved lines had at least 500, while L5 was the most mutated population with 2354 SNPs identified. This contrasted with the high host density worm evolved treatment group (Figure 3.15), which only contained 366 SNPs in its most mutated population (S6) while all remaining populations possessed less than 100 SNPs. Both control treatment groups displayed a similar level of total SNPs with all of the low host density controls (Figure 3.16) and the high-density controls (Figure 3.17) all containing between 200 and 250 SNPs. The only exception to this trend was S5C, which only possessed 52 SNPs. There was some correlation between *C. elegans* virulence and the total number of SNPs, particularly in the worm evolved populations, with lower numbers of SNPs correlating with worm virulence, as all worm evolved populations with less than 500 SNPs retained virulence. However, this pattern was not repeated for the control populations, which displayed both virulent and avirulent phenotypes, despite similar numbers of SNPs (Table 3.4).

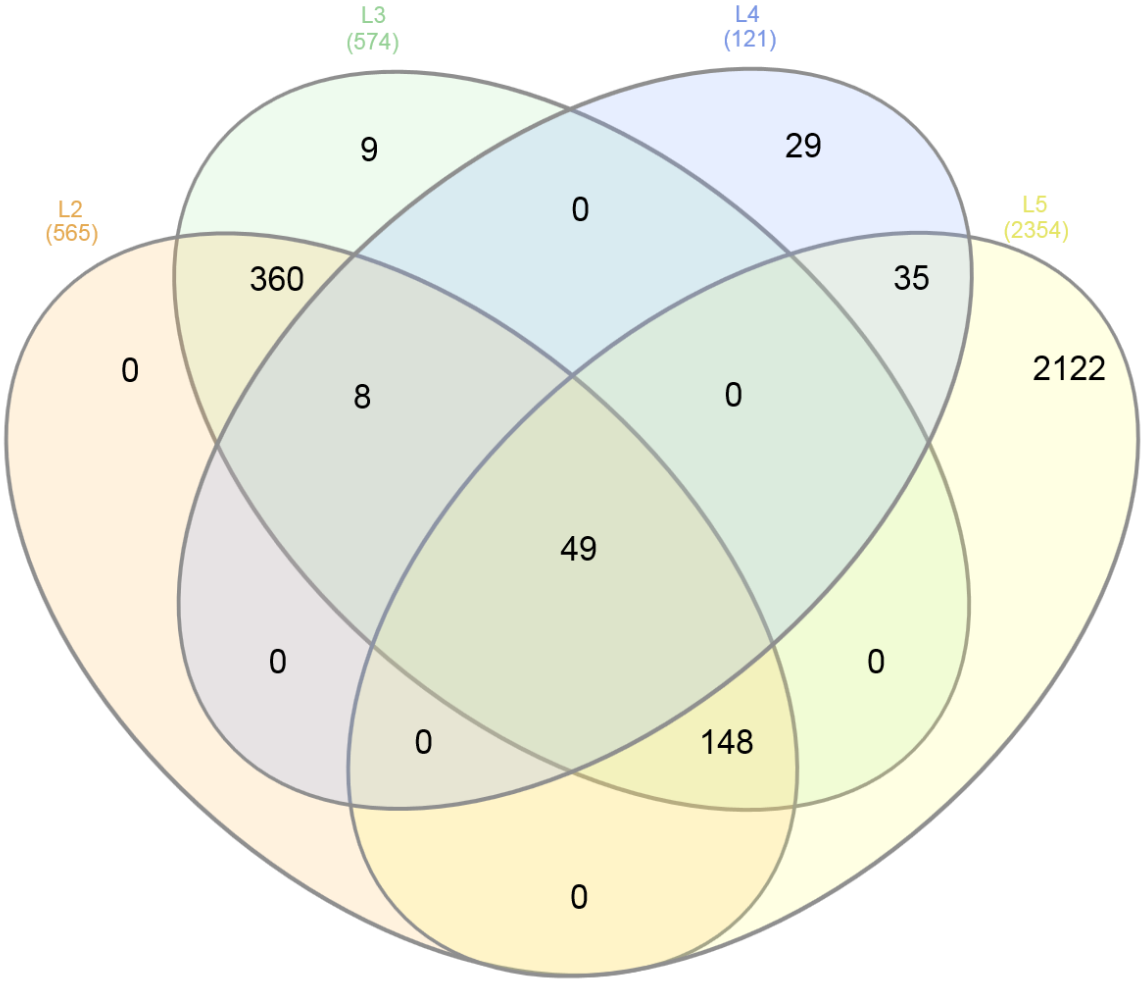


Figure 3.14: Total number of all SNPs found in the low host density worm evolved *P. aeruginosa* populations, and their overlap between populations of the same treatment group. The L4 populations contained the smallest number of SNPs (121) while L5 was the largest with 2354. L2 and L3 had 565 and 574 respectively.

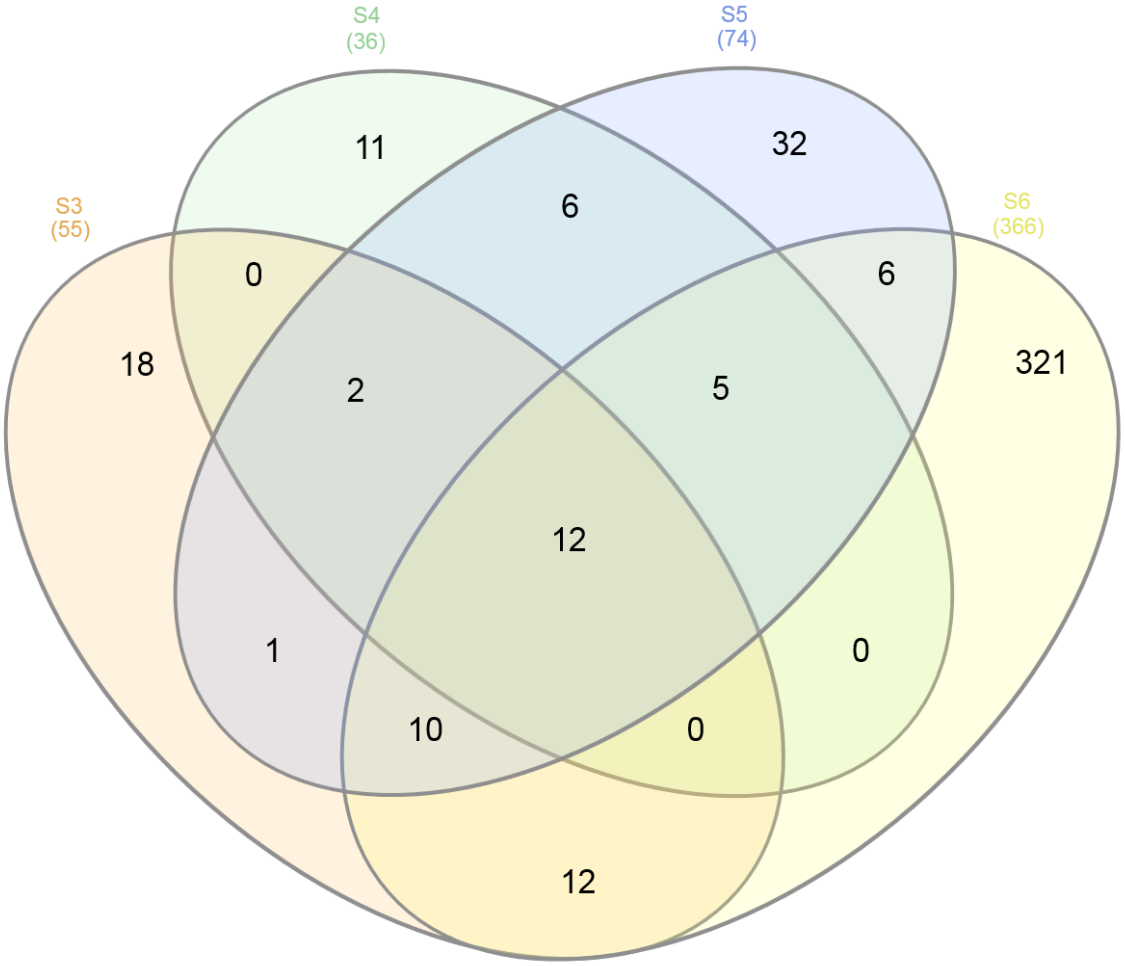


Figure 3.15: Total number of all SNPs found in the high host density worm evolved *P. aeruginosa* populations, and their overlap between populations of the same treatment group. The S6 population contained the largest number of SNPs with 366, followed by S5 with 74, then S3 with 55. S4 possessed the smallest number with only 36 SNPs detected.

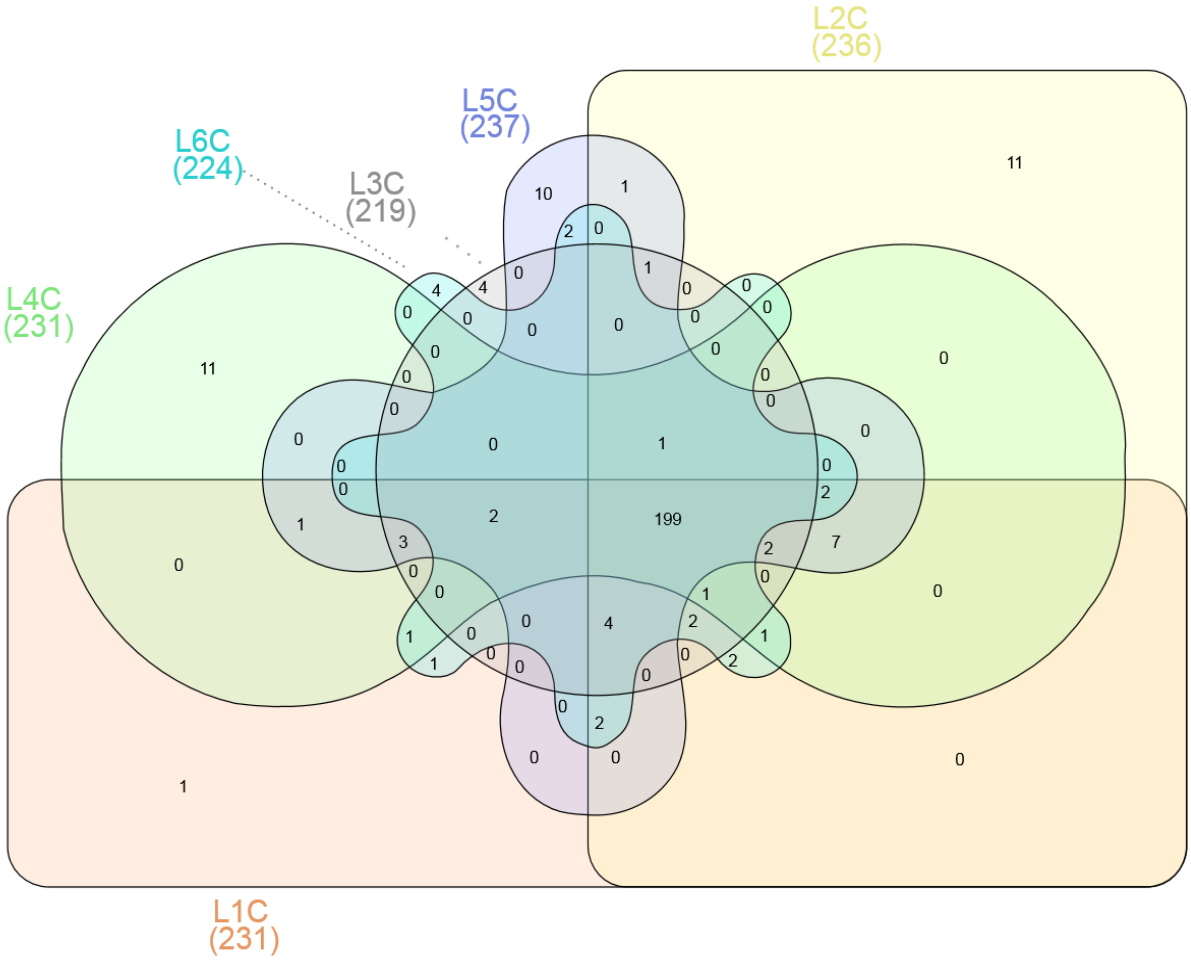


Figure 3.16: Total number of all SNPs found in the low host density control *P. aeruginosa* populations, and their overlap between populations of the same treatment group. All populations contained similar numbers of SNPs, all between 200 and 250. L5C contained the most, with 237. L2C was the next largest, with 236 L1C and L4C both possessed 231. L6C had 224 SNPs, leaving L3C with the least at 219.

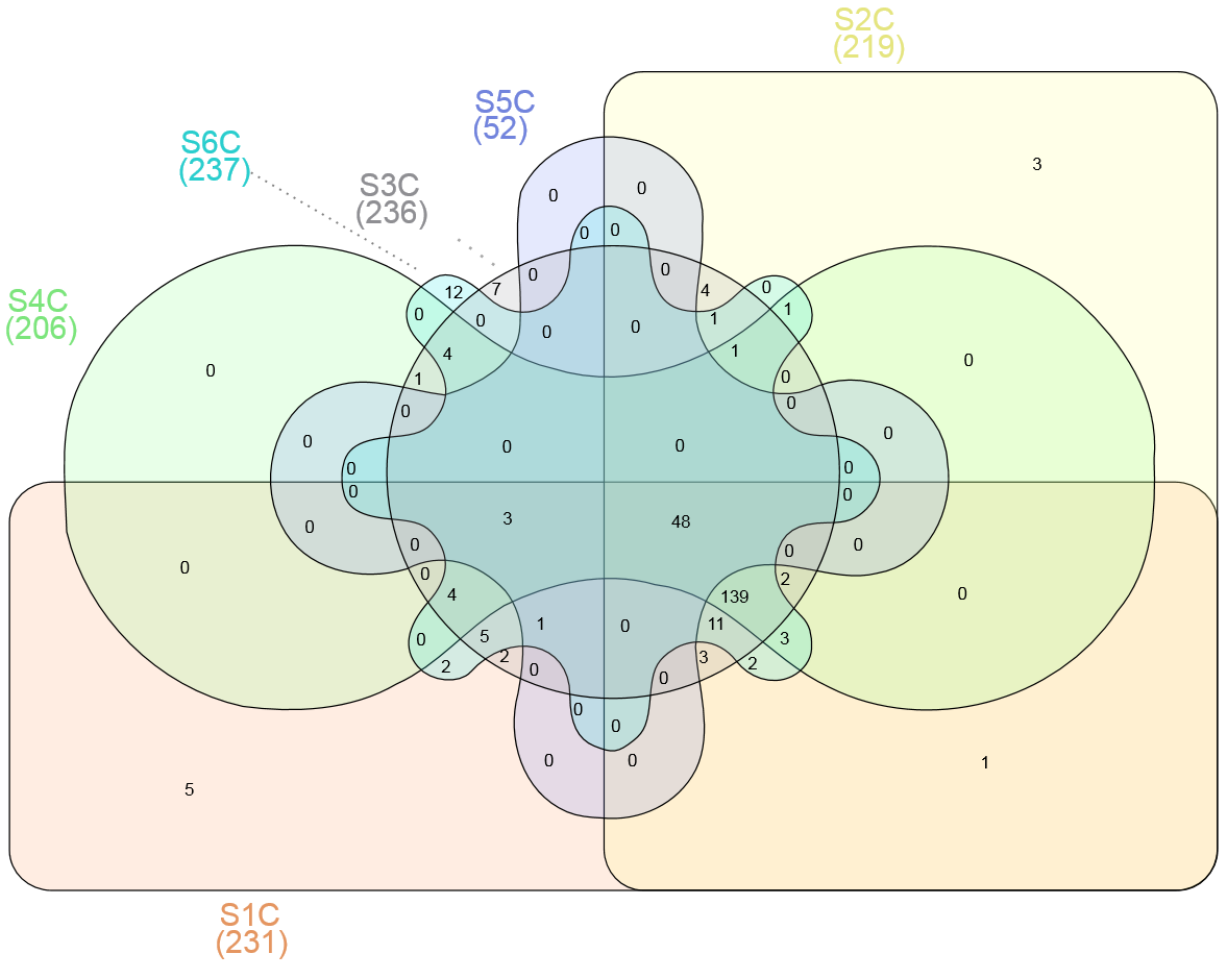


Figure 3.17: Total number of all SNPs found in the high host density control *P. aeruginosa* populations, and their overlap between populations of the same treatment group. The S6C population had the largest number of SNPs with 237, while S3C was similar with 236. The next largest was S1C with 231 SNPs. S2C contained 219 SNPs, followed by S4C with 206. The population with the smallest amount of SNPs was S5C, with only 52.

Population	Virulence	SNPs	Population	Virulence	SNPs
Worm Evolved					
L2	Negative	565	S3	Positive	55
L3	Negative	574	S4	Positive	36
L4	Positive	121	S5	Positive	74
L5	Negative	2154	S6	Positive	366
Controls:					
L1C	Negative	231	S1C	Positive	231
L2C	Negative	236	S2C	Positive	219
L3C	Negative	219	S3C	Positive	236
L4C	Positive	231	S4C	Positive	206
L5C	Negative	237	S5C	Positive	52
L6C	Negative	224	S6C	Negative	237

Table 3.4: Correlations between virulence towards *C. elegans* and number of SNPs in evolved *P. aeruginosa* populations. Virulence was only correlated for the worm evolved populations (L2-5 and S3-6), with virulence only retained in the populations with less than 500 SNPs (L4, S3, S4, S5 and S6). This correlation was not present in control populations (L1-6C and S1-6C), which all contained less than 500 SNPs regardless of virulence phenotype.

In addition to having the most total SNPs, the low host density worm evolved populations also possessed the largest amount of non-synonymous SNPs, both in number of genes covered and the number of SNPs in some specific genes (Figure 3.18). For example, the L5 population contained 39 functional SNPs in the genes *carB* and *acnA*, either of which is more than the total number of SNPs present in the least mutated population overall (S4). At least one population in the low host density worm evolved treatment groups contained a functional SNP in 109 different genes (Figure 3.18), while the low host density controls only demonstrated functional mutations in 41 (Figure 3.19). The difference between the high host density treatment groups was far closer, with 45 gene mutated in the worm evolved populations (Figure 3.20), and only 38 in the control populations (Figure 3.21).

Strikingly, there were few SNPs present in well established QS genes. Even *lasR* mutations were only present in very small numbers in the control populations (Figures 3.14 and 3.18), and were present in both virulent and avirulent populations. Overall, there were no clear patterns between specific SNPs and virulence across treatment groups. Some genes did correlate with virulence within treatment groups, such as *groL* in the low host density worm evolved group (Figure 3.15), with more SNPs present in avirulent populations. However, this correlation did not extend to the other groups, with *groL* absent from both groups of control populations (Figures 3.16 and 3.18), despite their varied virulence phenotypes and only present in one population (S6) of the high host density worm evolved group (Figure 3.20), despite those populations all retaining

worm virulence. Overall, there was no apparent association between the SNP patterns and virulence phenotypes.

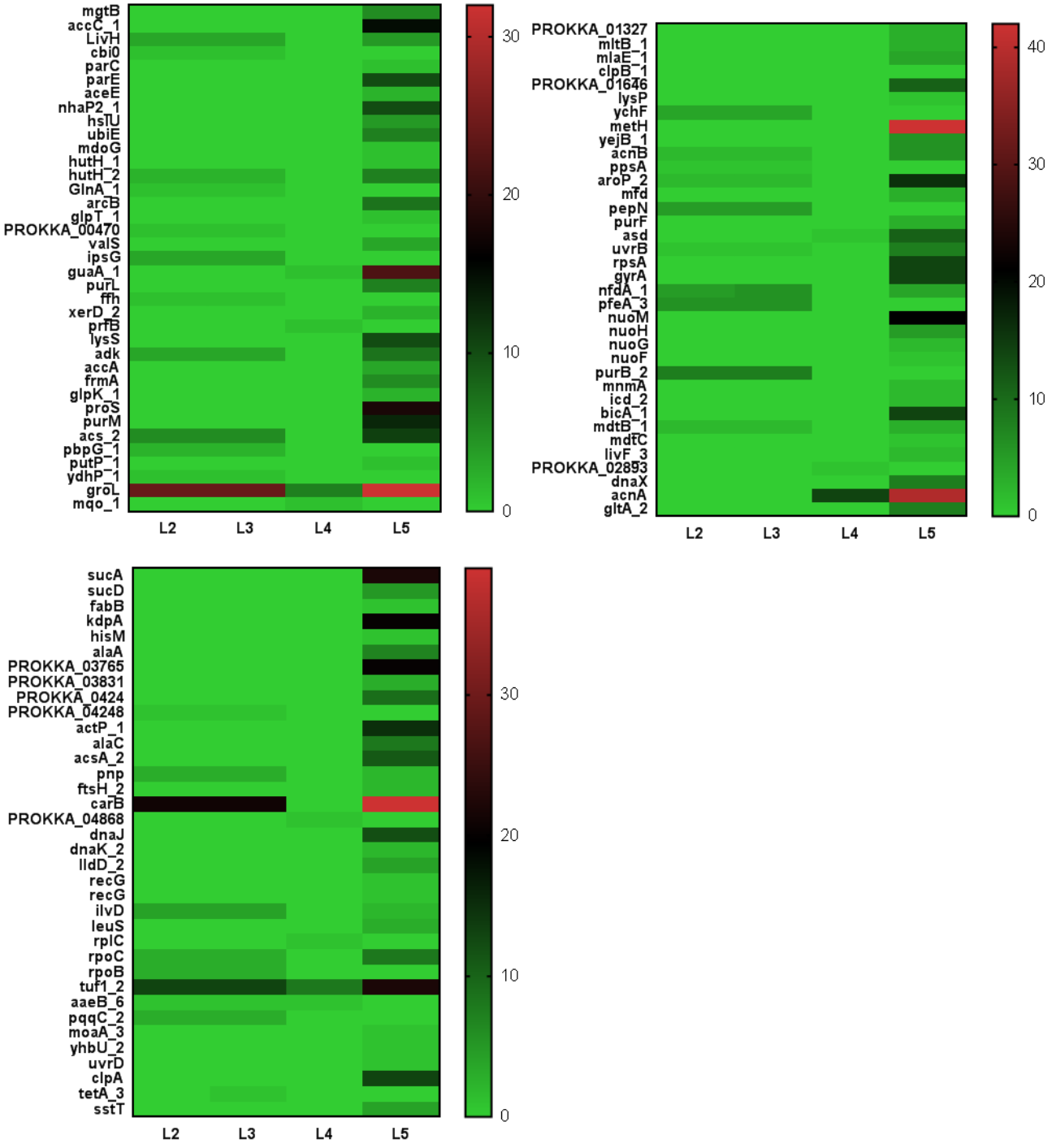


Figure 3.18: Heat map showing the locations of all functional SNPs in the low host density worm evolved *P. aeruginosa* populations, and the amount of SNPs present in each gene.

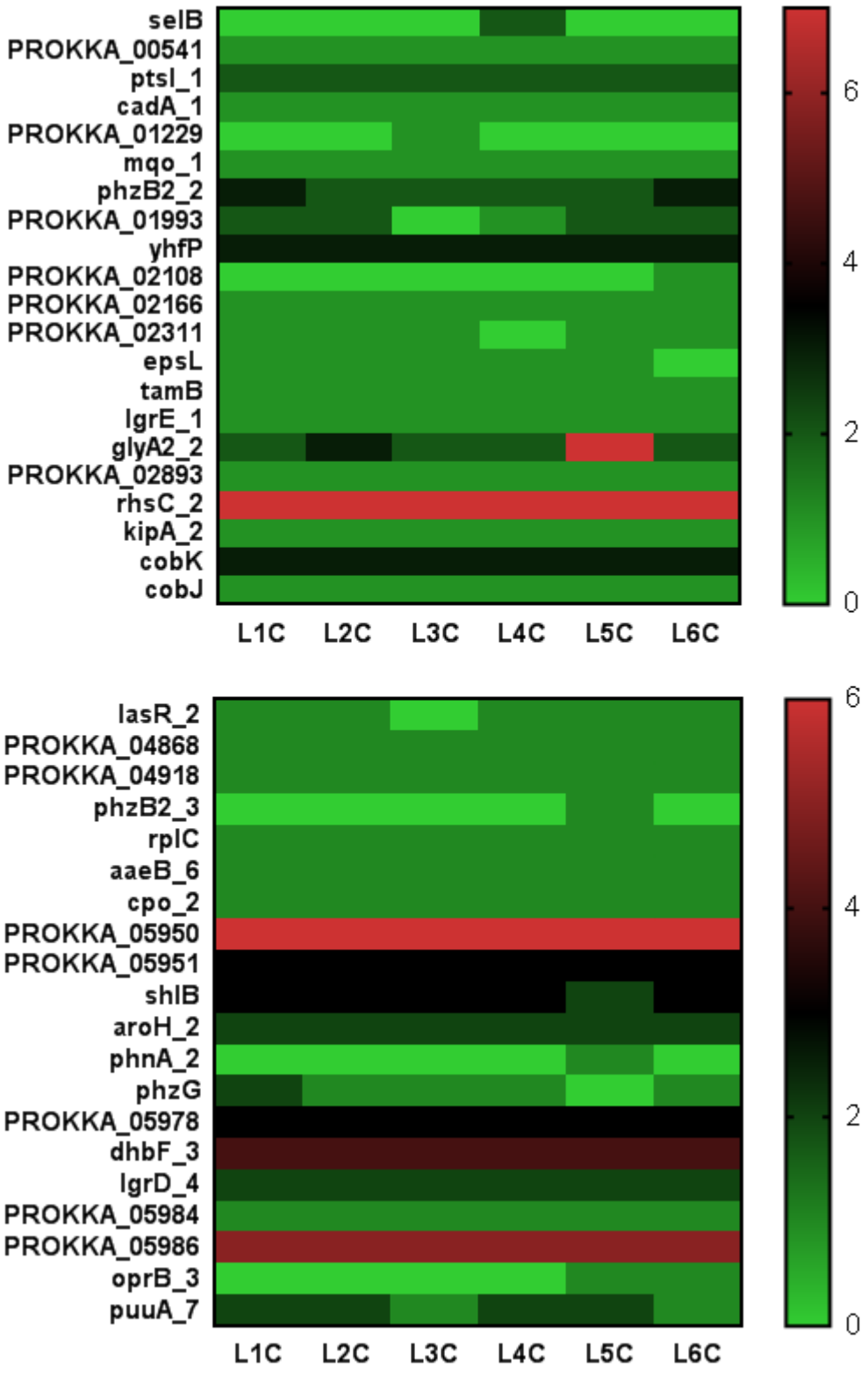


Figure 3.19: Heat map showing the locations of all functional SNPs in the low host density control *P. aeruginosa* populations, and the amount of SNPs present in each gene.

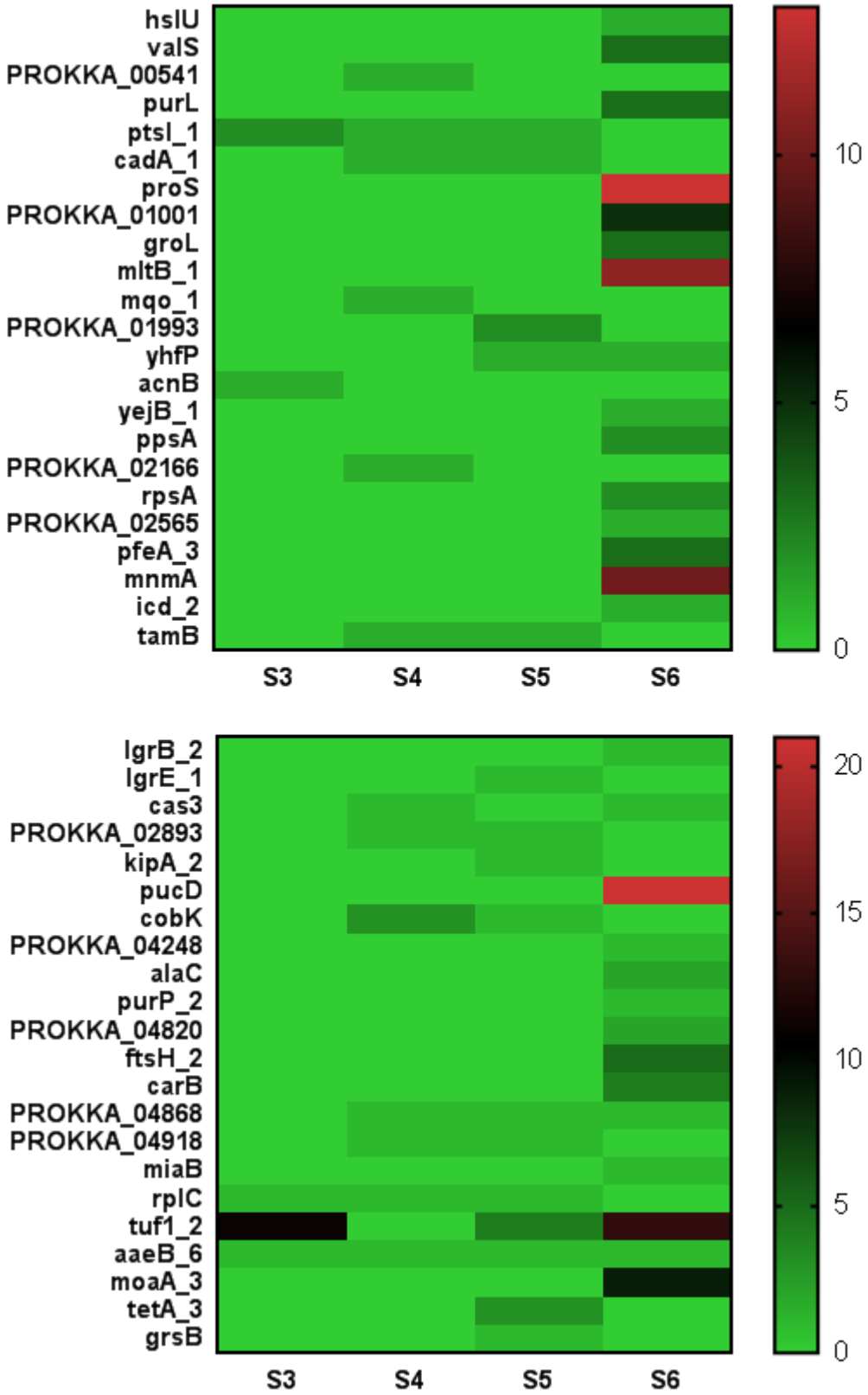


Figure 3.20: Heat map showing the locations of all functional SNPs in the high host density worm evolved *P. aeruginosa* populations, and the amount of SNPs present in each gene.

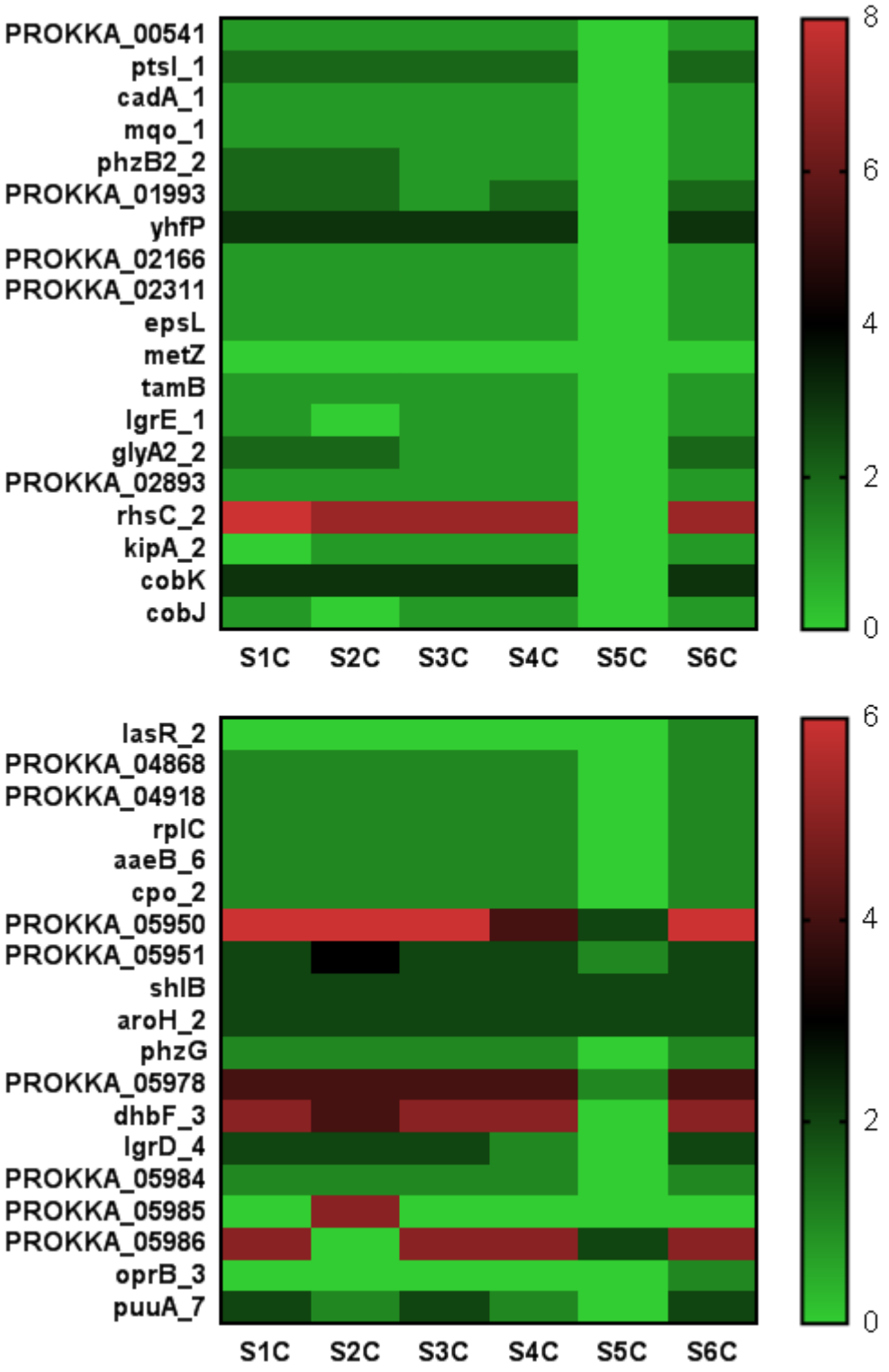


Figure 3.21: Heat map showing the locations of all functional SNPs in the high host density control *P. aeruginosa* populations, and the amount of SNPs present in each gene.

Discussion

A selection experiment protocol was devised and used to generate 20 populations of *P. aeruginosa*, 8 of which had evolved in a *C. elegans* host at different host densities, while 12 were control populations, evolved without the presence of worms. These populations were then tested for host killing, and virulence factor production and underwent whole genome sequencing to identify SNPs present at the population level. Additionally, 6 individual isolates were extracted from each evolved population. These 120 isolates underwent the same virulence factor assays as the populations. Unsurprisingly, evolved phenotypes did differ from the PA14 ancestor. However, the largest differences in phenotype observed were between bacteria evolved in the low host density and high host density environments, rather than between worm evolved and control groups. Siderophore production did not correlate with virulence towards *C. elegans*. However, several other virulence factors such as exogenous protease, pyocyanin, cyanide and QS signal molecules were correlated with *C. elegans* virulence, although several virulence factors together were needed to achieve worm virulence. Whole genome sequencing revealed a high level of variation in numbers of SNPs, even between populations from the same treatment group. However, there were no obvious links between the patterns of functional SNPs identified, and the virulence phenotypes of the bacterial populations. Even QS genes, which have repeatedly been highlighted as major factors in *P. aeruginosa* virulence[4, 84-87], did not contain functional mutation in the sequenced populations.

The virulence factor phenotypes detected here are somewhat in keeping with the established body of literature. Of the tested virulence factors, AHL signalling, pyocyanin, cyanide and protease proved to be correlated with worm virulence. Most of which have been individually linked with virulence in *C. elegans*[104, 160, 174]. However, the novel finding here is the extent to which virulence was dependent on a combination of these virulence factors, no single virulence factor was always present in virulent samples, and there were exceptions present for any correlated group. The lack of master regulation by AHL signalling in particular, is in keeping with the recent idea that quorum sensing in *P. aeruginosa* is an adaptable system, rather than a rigid hierarchy[16]. This finding highlights the robustness of *P. aeruginosa* as a pathogenic organism, with a wide array of potential virulence factors working together. Therefore, any future attempts to develop anti-virulence therapies, an attractive proposition in an environment of increasing antibiotic resistance[12], would benefit from a holistic examination of the entire virulence machinery, and avoid focussing only on single virulence factors.

The initial impression of the population level worm kill assays is that host density does play a role in the evolution of virulence. The high host density worm evolved populations, with their higher level of transmission opportunities, all retained virulence in *C. elegans* (Figure 3.6), while most of the low host density populations displayed heavily attenuated virulence (Figure 3.6). This would initially appear to confirm the hypothesis of the selection experiment, higher host densities and so increased transmission opportunities between hosts result

in retained or increased virulence. However, the results of the control populations challenge this conclusion. The low host density control population were very similar to the worm evolved, with more populations with attenuated virulence, but nevertheless a mix of retained and attenuated virulence was present (Figure 3.6). The high host density controls were even more similar to the worm evolved populations, with only a single population losing virulence (Figure 3.6). The difference in phenotypes observed in the two sets of control populations was surprising, as the only difference in their treatment was the size of petri dish used, which one would not expect to have an effect on a bacterial culture being manually sub cultured.

Indeed, the size of the petri dish used appeared to be the more important to the evolution of *P. aeruginosa* than the presence/absence of worms, with both the high density groups (using the large plates) and low density groups (using the small plates) showing more similarities to each other than between worm evolved and control groups. This suggests that the environmental conditions that result from a different sized petri dish can affect the evolution of *P. aeruginosa* populations more than the presence of *C. elegans*, and begs the obvious question of what is different to the bacteria about a different sized petri dish. Two possible explanations present themselves. Firstly, there may have been slight differences in the thickness of the growth media in the differently sized plates. If the smaller plates had a slightly thicker layer of media available, they may have found the richer growth environment easier to survive in. So, there would have been less evolutionary pressure to shed resource consuming and

unnecessary virulence factors. Whereas bacteria on the larger plates may have found their environment becoming resource depleted more quickly, which would have resulted in a competitive advantage for any mutants that could reduce their metabolic requirements. Secondly, the different sized environments caused by the two different plate sizes could result in bacterial populations with different spatial structures, with more diffuse populations present on the larger plates because there is more room for the bacteria to spread out. These different spatial arrangements will inevitably result in different evolutionary pressures, for example a mutant in a larger environment has the potential to expand with less direct competition from other evolving strain, as there is enough space and resources for both variants to persist. This could allow faster accumulation of mutants in the more diffuse communities, resulting in the observed differences in population level phenotype when all the variants are brought together for virulence assays. Under this explanation, it is possible that the bacterial populations from both plate sizes are heading towards the same end-point, adaption to life *in vitro* on a petri dish, and the populations growing in the more expansive environment of the larger petri dish are simply getting there faster than the more spatially compact populations from the smaller plates.

A similar pattern of virulence phenotypes was also present in the individual isolates assayed. This indicates a likelihood that there is no firm direction evolution will take on agar plates, it is unavoidably random. This stands in stark contrast to recent work that has claimed that *P. aeruginosa* will evolve to commensalism in *C. elegans*[174]. That study did not include any control

experiments, so its conclusion must be viewed as dubious in the light of the data presented here. The experimental protocol used also included a substantial amount of time in which the pathogen was grown independent of the host organism, and therefore was reproducing and evolving in the absence of the host for a proportion of the experiment. Another recent study on host-pathogen coevolution using a *C. elegans* host, which reported that virulence is favoured in coevolution, also cultured its 'host evolved' bacteria away from the host for regular periods of time[189]. The data presented here suggest that, unfortunately, the presence of the *C. elegans* host does not exert any important evolutionary pressures on *P. aeruginosa*. The observed evolved phenotypes being present in both the worm evolved and control populations would indicate that the phenotypes observed are the natural result of the general environmental conditions present in the experiment, not the host organism specifically.

Even if the previous work claiming *P. aeruginosa* can evolve to commensalism in *C. elegans*[174] is correct, it would still be of dubious use when considered in a human health context. It is claimed that *P. aeruginosa* achieves commensalism via the acquisition of *lasR* mutations. However, accumulation of *lasR* mutations is known to occur in the CF lung[94], yet *P. aeruginosa* remains a major chronic health concern to CF patients[95, 190, 191]. At best this would suggest that *C. elegans* is a poor host organism in which to study *P. aeruginosa* if the goal is insight into its role in human health, as the *lasR* mutations, though responsible

for attenuation in *C. elegans*[161, 174], have been linked with pulmonary inflammation and increased pathogenicity in chronic CF lung infections[95].

Several of the phenotypes tested across the different evolved populations did follow shared patterns. This was particularly striking with total protease and cyanide, which correlated fully. The cyanide/protease positive populations also correlated with higher levels of pyocyanin production. Even the L1C and L4C populations, which did display some attenuation of pyocyanin, still retained more activity than the protease/cyanide negative populations. This set of correlated virulence factor phenotypes also match the *C. elegans* killing data for the evolved populations. A similar pattern was observed in the isolate phenotypes, with the same set of 5 virulence factors, exogenous protease, cyanide, pyocyanin, C4-HSL and 3-Oxo-C12-HSL all being associated with virulence, and often being preserved together. Therefore, the presence of this battery of virulence factors appears to be an important aspect of the virulence of *P. aeruginosa* in *C. elegans*. However, none of these virulence factors turned out to be individually vital for virulence, as no single virulence factor fully correlated with worm killing activity. This result suggests that there is not a single 'master' virulence factor that controls *P. aeruginosa* killing *C. elegans*. Instead, it appears that virulence is a multifactoral process, where several of the virulence factors in the arsenal of *P. aeruginosa* are required to work in combination in order to achieve a lethal phenotype in the worm. This result runs contrary to the way a lot of virulence studies, not limited to those utilising *C. elegans,* have reported their findings, which focus on single virulence factors to which a high importance is

attached[1, 84, 99, 101, 160, 161]. Even AHL-mediated QS signalling, often presented as a key element of virulence[108, 161, 162], was not universally correlated, with examples of both AHL retention, but no worm killing (S41), and a loss of AHLs, but preserving worm killing (S5C3). This particular result is in keeping with a recent publication, which claims that the *P. aeruginosa* QS system may be more robust than previously thought, with greater redundancy and ability to adapt around individual loss of function mutations[16]. This is in keeping with the picture of virulence presented by this study, a system of marginal contributions by several virulence factors, which combine to form an overall virulent phenotype. While QS may indeed still play a vital role in this process, it does not appear to be inextricably linked with the production of either AHL assayed here. The lack of a genomic basis for the phenotypes observed, particularly given the presence of what appear to be QS negative strains, suggests the focus of research on QS signalling mechanisms may be a dead end, as the bacteria appear to have a diverse and durable system of virulence regulation, as even the signal molecules themselves were not ultimately necessary for virulence.

Siderophore production, on the other hand, is more random, with some populations, such as L4, having greater loss of pyoverdin than pyochelin, while others, such as L2, display the opposite, and, even within the same population group, such as L5, just lose some activity in both siderophores. This result would seem to indicate that siderophore production is not an important factor in the *P. aeruginosa* life cycle under in vitro conditions, or in the *C. elegans* host, as no

strong trends of altered siderophore activity developed during the selection experiment. This may be due to a sufficient iron presence in the environment, so there is no need for siderophores to promote virulence to liberate additional iron from host proteins (a suggested explanation for pyoverdin's role in virulence[99]). Therefore, it appears that siderophores are not an important virulence factor in *P. aeruginosa* virulence towards *C. elegans*. This result contradicts a previous study, which reported pyoverdin as an indispensible virulence factor for *P. aeruginosa* in the *C. elegans* host[99]. However, this discrepancy may be due to differences in experimental protocol, such as the previous study using a liquid-killing assay under high humidity, in contrast with the plate-based assays used in this study, or differences in iron levels in the respective growth media.

The lack of obvious patterns in the sequencing data could be due to the complex nature of *P. aeruginosa* virulence, which does employ a wide array of virulence pathways[1, 4, 81, 93, 97-99, 104, 105, 109, 114, 141, 161]. That there are enough redundancies and alternate pathways that no specific gene is a 'master controller' of virulence, and that reduced virulence is the result of a variety of marginal attenuations in the total virulence machinery. There was even a lack of SNPs in expected genes, such as *lasI/R* and *rhlI/R.* While initially surprising, given the reduced virulence phenotypes present in some of the evolved populations, this result may illustrate the way that *P. aeruginosa* virulence is not under the influence of only a handful of genes. Just as the recent finding that the QS system in *P. aeruginosa* is more robust than previously thought, suggesting that multiple

redundant pathways are involved, even in classically QS-mediated areas[16]. So the genetics of *P. aeruginosa* virulence may be far more intricate and complicated than the single point regulation explanations which have dominated thought on the subject thus far[192, 193]. Additionally, the heterogenous nature of the populations may be a factor. Not only are there no guarantees that every SNP in the population was picked up by the sequencing reaction, it also could not provide an indicator of the frequency of SNPs within the population. So, a population such as L5, with an anomalously high number of SNPs, could be the result of a rare, highly mutated variant being caught in the sequencing screen. Likewise, the paucity of SNPs in S5C compared to the rest of its treatment group could ultimately be due to the selection of mutants caught and included in the sequencing reaction, which may not have been identical to the selection and relative proportions used in the virulence and worm kill assays. Unfortunately, that is an unavoidable side effect of using these bacterial populations.

Alternatively, the answer may lie in the array of currently uncharacterised genes, signified by the PROKKA title and relevant reference number. As their function is unknown, the mutations in such genes could ultimately be the root cause of the divergent phenotypes observed in the evolved populations. However, the characterisation of this battery of genes goes beyond the scope of this study.

Overall the selection experiment can be viewed as reasonably successful. Twenty rounds of passage through *C. elegans* was able to generate unique heterogenous populations of *P. aeruginosa* from the PA14 ancestor. Strangely, the biggest similarities were between the 2 low host and the 2 high host density populations,

rather than between worm evolved and control treatment groups. Therefore, the initial hypothesis that low host density conditions would promote a shift towards reduced virulence of *P. aeruginosa* cannot be confirmed based on these data. The virulence factor assays did show that *P. aeruginosa* virulence is multifaceted, and that a combination of several virulence factors, including exogenous protease, cyanide, pyocyanin and AHL activity is needed to enable the killing of *C. elegans*. However, the genetic alterations underpinning the observed phenotypic changes remain elusive, despite the sequencing. Of all the possible explanations for this, the most likely is that virulence attenuation is the result of many marginal reductions rather than one or two key mutations.

Chapter 4: Assessing variations in *Pseudomonas aeruginosa* virulence across multiple host organisms

Abstract

Pathogenic bacteria evolve alongside their host organism, with the nature of that evolution dependent on the specifics of the host-pathogen interaction. However, opportunistic pathogens such as *P. aeruginosa* have a wide variety of possible host organisms to infect, with potentially varying levels of virulence between hosts. This chapter tests the extent to which *P. aeruginosa* virulence is preserved or differs across different host organisms. To contrast the virulence *P. aeruginosa* displays towards *Caenorhabditis elegans* (see Chapters 2&3), two additional hosts were used, waxmoth larvae (*Galleria mellonella*) and mung bean seedlings (*Vigna radiate*). A substantial variation in virulence between hosts was observed. While there was some acute variation in virulence towards *G. mellonella*, all *P. aeruginosa* strains and populations tested were highly virulent, resulting in the rapid death of larvae. The opposite occurred for *V. radiate*, with no *P. aeruginosa* strain displaying observable virulence towards the plants. These contrast with the highly varied levels of virulence in a *C. elegans* host described in the previous chapters. Therefore, for an opportunistic pathogen such as *P. aeruginosa*, the type of host plays a major role in whether a virulence phenotype is displayed.

Introduction

Opportunistic pathogens, such as *P. aeruginosa*, can grow in a wide variety of environmental niches which contrasts strongly to specialist primary pathogens. A primary pathogen's life cycle is dependent on colonisation of a specific host[194], and therefore will only interact with a small selection of environments, such as the in-host environment, reservoir environment, and potentially a vector environment. An opportunist, on the other hand, will encounter a wider variety of host environments, as opportunists will often be capable of exploiting multiple hosts[79], and each new host will have a unique set pathogen interactions and likely a different virulence phenotype. As *P. aeruginosa* encounters, and is potentially pathogenic towards, multiple organisms, a comparison of virulence across different hosts would highlight the influence hosts can exert on host-pathogen dynamic[7-9, 11], and help with the correct choice of host models in future study design.

The greater wax moth, *Galleria mellonella*, is an insect of the family *Pyralidae*, order *Lepidoptera*[195]. The larvae of this species have recently been gaining popularity as an animal model. Although the use of *G. mellonella* as a model goes back to the 1970s[196], it has experienced a recent upsurge in popularity. Of the more than 1000 articles published on PubMed about the *G. mellonella* infection model, over 200 were published in 2014-2015[197]. Compared to traditional mammalian host models such as mice, *G. mellonella* has a number of advantages that make it attractive for use as a host organism. It is significantly cheaper, as the larvae are commercially available through pet food retailers. Additionally, no

special lab equipment is required to maintain a population[198]. Their use does not require ethical approval. The short lifespan of the larvae easily accommodates their use for high-throughput studies.

Although insects and vertebrates have massively diverged over many millions of years, some important similarities remain. Although insects, *G. mellonella* included, did not develop an adaptive immune response, the immune response which they do possess retains substantial similarities with the mammalian innate immune response[199], allowing for the use of *G. mellonella* in the study of both fungal and bacterial infections, as well as a testing ground for the efficacy of novel drugs[197]. When used to study *P. aeruginosa*, positive correlation between virulence in *G. mellonella* and mice has been reported[200], solidifying this model as a clinically relevant tool.

The majority of studies performed with *G. mellonella* have utilised injection of live bacteria into the larvae, with virulence assessed by checking survival rates at different time periods with assays lasting up to 5 days[197]. A wide variety of bacteria have been used in this model, including both Gram-positive and negative species. Gram-positive species include, *Streptococcus pneumoniae*[201], *Listeria monocytogenes*[202-206], *Enterococcus faecalis*[207] and *Staphlococcus aureus*[208-210]. Gram-negative species used include *Legionella pneumophila*[211], *Escherichia coli*[212-214], *Klebsiella pneumonia*[215-218] and *Pseudomonas aeruginosa*([196, 200, 219-231]. *P. aeruginosa* in particular has been heavily studied, going back over forty years[196]. *P. aeruginosa* has been

shown to be highly virulent in *G. mellonella*, with some strains being lethal within 24 h of exposure using bacterial loads as low as 10 live cells[227].

The use of plant models to study *P. aeruginosa* virulence is also well established. Similar patterns of virulence factor expression have been observed in both plants and mice[146, 232]. Although several plant models have been documented, including basil (*Ocimum basilicum*)[233] and lettuce (*Lactuca sativa*)[146], the best established is a leaf model using *Arabidopsis thaliana*[182, 232-236]. While Arabidopsis is a well established model, with a sequenced genome and library of available mutants to test[235], and is known to display different virulence phenotypes when infected with mutant *P. aeruginosa*[233], the model focuses on the leaves of the plant. Therefore, there are aspects of the bacteria-plant relationship that the Arabidopsis model does not account for, such as the infection of roots or young plants in soil, which may be closer to how the bacteria infect plants. An assay utilising sprouted mung beans (*Vigna radiate*) was developed in part to test interactions in this different plant-bacteria environment, as well as providing another plant model as a tool for the study of bacterial virulence.

A previous study identified and extracted several species of bacteria, including pseudomonads, from spoiled commercial bean sprouts[237]. They went on to show a loss of AHLs, and concurrent loss of the virulence controlled by the signal molecules, preventing the spoilage of the sprouts. This raised the possibility that mung beans could be used as a viable host organism to study *P. aeruginosa* in a clinical context. Like the other extant plant models, it is both cheap to use, and

carries no ethical implications. Initial trials of a mung bean assay presented results indicating its suitability in this capacity (Garge, Unpublished data).

Having established the behaviour of evolved *P. aeruginosa* in the host it had evolved in, *C. elegans*, (see Chapter 3) the question became whether changes in virulence levels were conserved across multiple species of host. The aim of this chapter was to establish whether *P. aeruginosa* virulence is specific to the host species it evolves in, or whether virulence varies between species. Virulence proved to vary drastically between hosts. While the different *P. aeruginosa* strains tested had previously displayed a variety of virulence phenotypes in the *C. elegans* host, the two additional hosts were more uniform, with universal high virulence and rapid larvae death in *G. mellonella*, while the bacteria were uniformly avirulent in *V. radiate*.

Materials and Methods

Strains used in this study

Strain	Description	Source
***Escherichia coli*:**		
OP50	Uracil auxotroph. Used as food source for *C. elegans* and as a negative control. Tetracycline^{R} (Tc)	Epstein & Shakes (1995)[166]
***Pseudomonas aeruginosa*:**		
PA14	PA14 wild type.	Rahme et al (1995)[146]
PA14 Δ*lasR*	PA14 with a *lasR* insertion mutation.	Rumbaugh et al (2009)[167]
PA14 Δ*lasI*	PA14 with a *lasI* insertion mutation.	Rumbaugh et al. (2009)[167]
PA14 Δ*rhlR*	PA14 with a *rhlR* insertion mutation.	Rumbaugh et al (2009)[167]
PA14 Δ*rhlI*	PA14 with a *rhlI* insertion mutation.	Rumbaugh et al (2009)[167]
PA14 Δ*pqsE*	PA14 with an unmarked *pqsE* deletion.	Steve Diggle Strain Collection, unpublished
PA14 Δ*pqsR*	PA14 with an unmarked *pqsR* deletion.	Steve Diggle Strain Collection, unpublished
PAO1-N	PAO1 Nottingham wild type.	The Holloway Collection
PAO1-N Δ*pqsA*	PAO1-N *pqsA* deletion mutant.	Aendekerk et al (2005)[168]
PAO1-N Δ*pqsE*	PAO1-N *pqsE* deletion mutant.	Rampioni et al (2010)[169]
PAO1-P	PAO1 USA Matt Parsek wild type.	Holloway et al (1979)[170]
PAO1-P Δ*pelA*	PAO1-P *pelA* mutant, polar knockout of *pel* operon.	Borlee et al (2010)[171]
PAO1-P Δ*pslBCD*	PA01-P *pslBCD* mutant, polar knockout of *psl* operon.	Kirisits et al (2005)[172]
PAO1-P Δ*pel/psl*	PAO1-P *pelA/pslBCD* double mutant.	Borlee et al (2010)[171]

Strain	Description	Source
CW2 B1	Clinical Isolate from Chronic Diabetic Ulcer Wound, taken from patient's bone.	Ana DeSilva, The University of Nottingham, Unpublished.
CW3 T1	Clinical Isolate from Chronic Diabetic Ulcer Wound, taken from patient's tissue.	Ana DeSilva, The University of Nottingham, Unpublished.
CW3 B1	Clinical Isolate from Chronic Diabetic Ulcer Wound, taken from patient's bone.	Ana DeSilva, The University of Nottingham, Unpublished.
CW4 T1	Clinical Isolate from Chronic Diabetic Ulcer Wound, taken from patient's tissue.	Ana DeSilva, The University of Nottingham, Unpublished.
CW5 T1	Clinical Isolate from Chronic Diabetic Ulcer Wound, taken from patient's tissue.	Ana DeSilva, The University of Nottingham, Unpublished.
CW5 T2	Clinical Isolate from Chronic Diabetic Ulcer Wound, taken from patient's tissue.	Ana DeSilva, The University of Nottingham, Unpublished.
SED2	Clinical Cystic Fibrosis Sputum Isolate Haplotype 2 (Darch15)	Darch et al (2015)[173]
SED6	Clinical Cystic Fibrosis Sputum Isolate Haplotype 6 (Darch15)	Darch et al (2015)[173]
SED8	Clinical Cystic Fibrosis Sputum Isolate Haplotype 8 (Darch15)	Darch et al (2015)[173]
SED16	Clinical Cystic Fibrosis Sputum Isolate Haplotype 16 (Darch15)	Darch et al (2015)[173]
SED20	Clinical Cystic Fibrosis Sputum Isolate Haplotype 20 (Darch15)	Darch et al (2015)[173]
SED22	Clinical Cystic Fibrosis Sputum Isolate Haplotype 22 (Darch15)	Darch et al (2015)[173]
AN1	PA14 wild type containing a *lasB:lux* promoter fusion.	This Study
Evolved *P.*	Selection of heterogenous	This Study

Strain	Description	Source
aeruginosa isolates	populations and individual isolates evolved during the selection experiment performed in Chapter 3 of this study. A full list is provided in Appendix 2.	

Table 4.1: List of bacterial strains used in this study.

Galleria Assay

Bacterial strains were cultured overnight in liquid slow kill (SK) media (3.5 g l^{-1} Peptone, 3 g l^{-1} NaCl, 1 l^{-1} ml Cholesterol (5 mg ml^{-1} in ethanol), 1 ml l^{-1} 1M $CaCl_2$, 1 l^{-1} ml 1M $MgSO_4$ and 25ml l^{-1} 1M KH_2PO_4) at 22°C. They were then filter sterilised using a 0.2 µm filter and the supernatants frozen until the larvae were available. *Galleria mellonella* larvae were acquired from www.wigglywigglers.co.uk. Each larva was injected with a 50 µl aliquot of the thawed supernatant between the rear two sets of legs and left in a compartment of a 28 compartment pill box at room temperature. A Tridak Stepper STP4001-0010 was used for the injections to ensure an equal volume of supernatant was administered to each larva. After the appropriate time the larvae were observed, looking for colour change, partial paralysis, fluid response and death to ascertain the virulence of the injected supernatant. Measurements were taken at 1 h and 48 h. Details of the injection site and phenotypes observed are illustrated in Figure 4.1.

The observed phenotypes of larvae were then used to extrapolate two measures of virulence: survival rate and infection index. Survival rate was simply the

percentage of larvae left alive at the time measured. Though a numeric infection index has previously been published for *Streptococcus pyogenes* in *G. mellonella* larvae[238], this index was not suitable for use with *P. aeruginosa* as it included phenotypes absent in the performed assay, such as cocoon formation, while omitting a phenotype present in many of the *P. aeruginosa* samples assayed, the fluid response. Therefore, an alternate infection index was implemented, in which the phenotypes of all tested larvae were assigned numeric values and totalled, resulting in a number for each strain, isolate or population where a higher number indicated higher virulence. Living larvae contributed +0 to the index, colour change, partial paralysis and fluid response contributed +1 each, while death added +2 to the index.

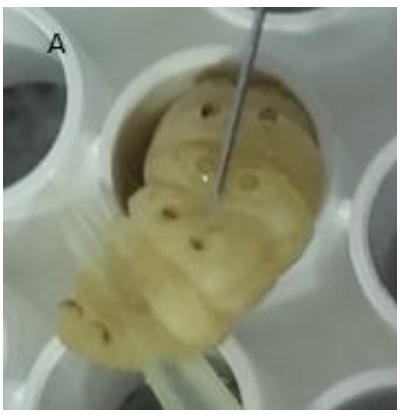








Figure 4.1: *G. mellonella* larvae images.
A: Injection site and illustration of restraining the larvae.

B: Fluid Response Phenotype.

C: Dead phenotype.

D: Alive phenotype

Mung Bean Model

Mung Beans were sterilised by soaking them in 100% ethanol for a minimum of 15 minutes. They were then removed from the ethanol, thoroughly rinsed with sterile water and transferred to a 0.8% agar plate. To ensure adequate hydration during the germination process, 2 ml sterile water was also added to the plate. The beans were then incubated at 37°C under humidified conditions for 24 hours to allow the beans to germinate.

Murashige and Skoog agar was prepared (4.5 g l^{-1} Murashige and Skoog Basal medium, 8 g l^{-1} agar) in 200 ml aliquots in 400 ml flasks. Overnight cultures of the bacteria to be tested were pelleted by centrifugation at 1664 rcf for 30 minutes and resuspended in 5 ml sterile water, 10 germinated beans were then added to each culture and incubated under static condition at 30°C to infect the beans. Negative control samples were prepared with distilled water in place of the concentrated bacterial culture. The infected, germinated mung beans were then added to each flask and allowed to grow under natural light conditions for 7 days. To ensure sterile aeration each flask was secured with a 0.2 µm filter. After 7 days the plants were removed from the flasks and assessed for mortality, shoot length, root length and number of root branches.

Statistical Analysis

Statistical analysis of all acquired data was performed using Graphpad Prism 7, http://www.graphpad.com/scientific-software/prism/).

Results

P. aeruginosa is rapidly lethal towards *G. mellonella*

Two measures of virulence were performed, percentage survival and infection index. However, as they were essentially in agreement, only the percentage survival data is presented here. Infection Index data is provided in Appendix 2.

The *P. aeruginosa* supernatants were highly virulent to the moth larvae, without exception. All tested strains resulted in 100% lethality in the moth larvae within 48 h of exposure. However, when examined after only 1 h, some variation could be observed.

Of the characterised lab strains and mutants tested in *G. mellonella* after 1 h exposure to bacterial supernatant, wild type PA14 proved to be the most virulent, a 0% survival rate (Figure 4.2). Although both tested PAO1 wildtype strains also demonstrated high levels of virulence in this host organism, neither possessed the complete lethality seen in PA14.

The PA14 mutants had reduced virulence compared to the wild type, and they displayed a variety of virulence levels. PQS mutants retained the most virulence, while most of the *las/rhlI* mutants adopted phenotypes of intermediate virulence. Only a single mutant, *ΔlasR* achieved the complete lack of virulence seen in the PBS and OP50 negative controls.

The PAO1 mutants were more homogenous. For PAO1-N *ΔpqsA* did have a higher infection index, despite the same % survival rate, while *ΔpqsE* was the opposite, with a slightly higher % survival rate, yet a lower infection index.

Results for PAO1-P were similar. Although *ΔpelA* had a lower % survival rate, it retained an identical infection index to the wild type. Both of the other polysaccharide mutants had a slightly reduced infection index, yet only the *Δpel/psl* mutant also had a reduction in survival rate. However, most of these variations were in reality only small changes, suggesting that these mutations are not having drastic effects on the virulence of *P. aeruginosa* PAO1 strains towards the moth larvae.

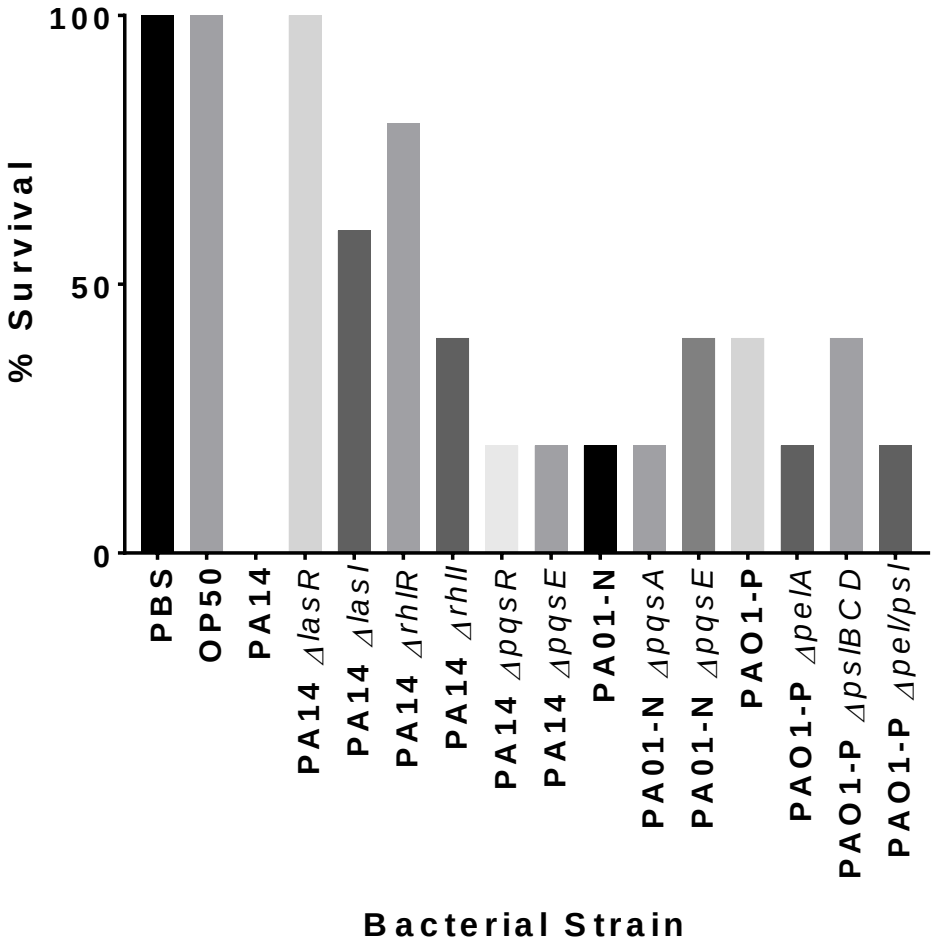


Figure 4.2: Survival of *G. mellonella* 1 h after injection with *P. aeruginosa* laboratory wild types and mutant supernatants. 5 replicates per strain.

There was no single virulence phenotype that predominated for either of the sets of clinical isolates tested. The chronic wound isolates had the widest variation of virulence phenotypes (Figure 4.3). Some isolates, CW2 B1 and CW5 T1, displayed the lowest possible virulence, with 100% survival. In contrast CW5 T2 had very high virulence, achieving 100% lethality. The remaining 3 isolates in the group were all of intermediate virulence, with % survival rates of 40-60%. The cystic fibrosis isolates were not so varied. Although some variation was present, this isolate group all trended towards higher virulence, with half the tested isolates, SED2, SED8 and SED16 all achieving 0% survival. While the other 3 isolates did not reach such extremes, SED20 and SED22 were both still more virulent than any of the chronic wound 'intermediate' isolates, and SED6, the least virulent cystic fibrosis isolate, was still at the same level as the most virulent intermediate chronic wound isolate, CW3 B1.

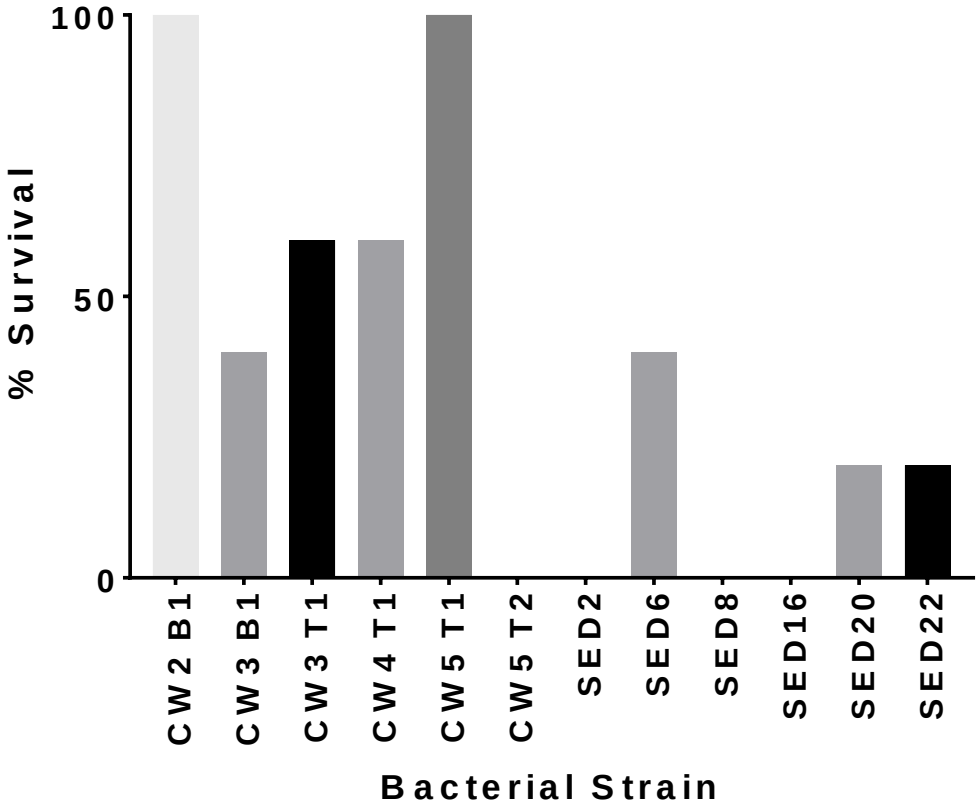


Figure 4.3: Survival of *G. mellonella* 1 h after injection with *P. aeruginosa* clinical isolate supernatants, derived from diabetic chronic wounds (CW) and cystic fibrosis sputum (SED), 5 replicates per strain.

The majority of the low host density worm evolved populations displayed relatively low virulence towards *G. mellonella* larvae. The only exception was the L4 population, which had an intermediate survival % (Figure 4.4). This was substantially different to the high host density worm evolved populations. Although only 2 of the populations, S3 and S6, achieved 100% lethality, the remaining 2 still demonstrated intermediate levels of virulence, which were consistent with that of the ancestor strain. This treatment group also lacked any examples of 100% survival seen in the low host density group. Both control treatment populations displayed a wide variety of virulence phenotypes, however the high host density controls did trend more towards virulence than the low host density controls. Although 100% survival was present in both groups, only the high host density group contained examples of 0% survival, the deadliest low host density populations having 20% survival.

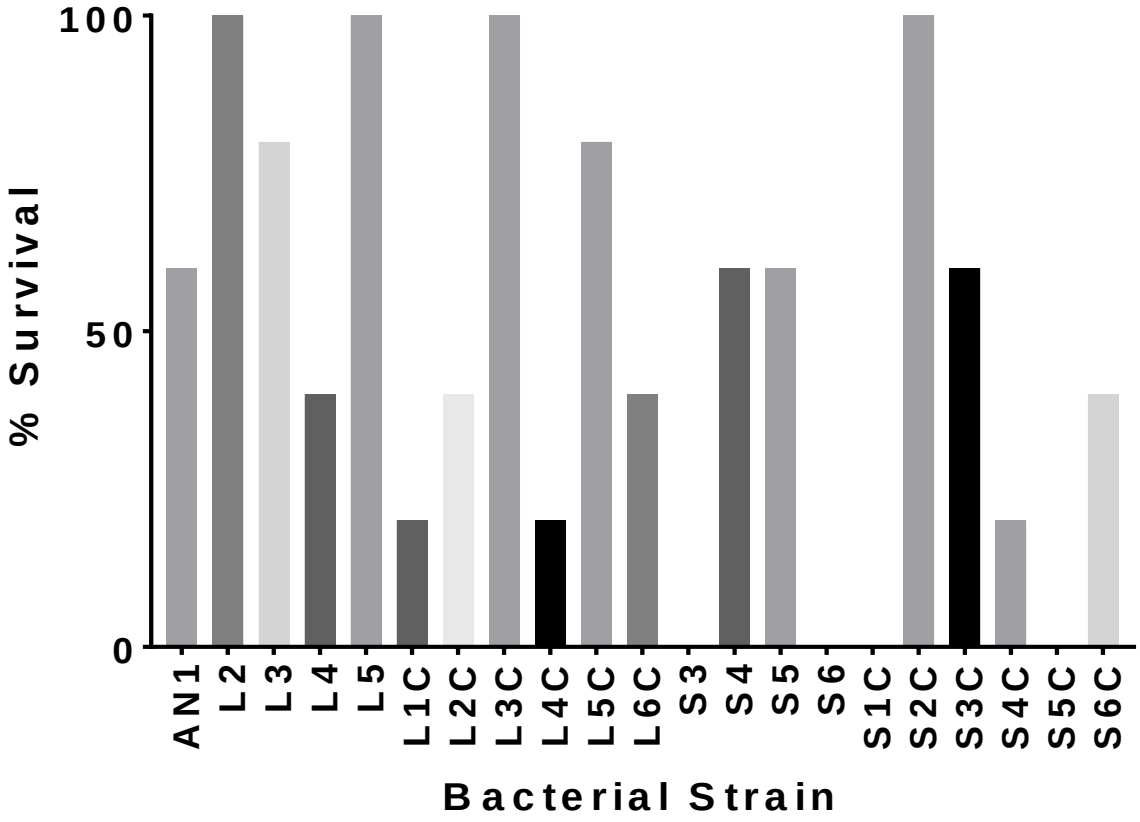


Figure 4.4: Survival of *G. mellonella* 1 h after injection with *P. aeruginosa C. elegans* evolved population supernatants, 5 replicates per population. AN1 is the Ancestor strain, L denotes low host density worm evolved conditions, LC are low host density controls, S are high host density worm evolved and SC and high host density controls.

No pattern of virulence emerged when the low host density worm evolved isolates were tested in the *G. mellonella* larvae. All population groups contained examples of both high and low virulence (Figure 4.5). Most population groups contained examples of 100% larvae survival (L21, L32, L51, L53). More populations were lacking extremely lethal isolates, both the L4 and L5 populations did not contain any 100% lethal isolates, bottoming out at 20% (L44) and 40% (L55) respectively. Despite this lack of high virulence, L4 isolates also lacked any examples of 100% survival, all isolates retaining at least virulence towards the larvae. It was a similar story for the low host density control isolates (Figure 4.5). Although most of the control population groups did not contain examples of 0% survival, such isolates only being present in the L4C and L6C groups, high and low virulence isolates were present in all groups. Unlike the worm evolved samples, every population in this treatment group did contain at least one isolate with the 100% survival phenotype.

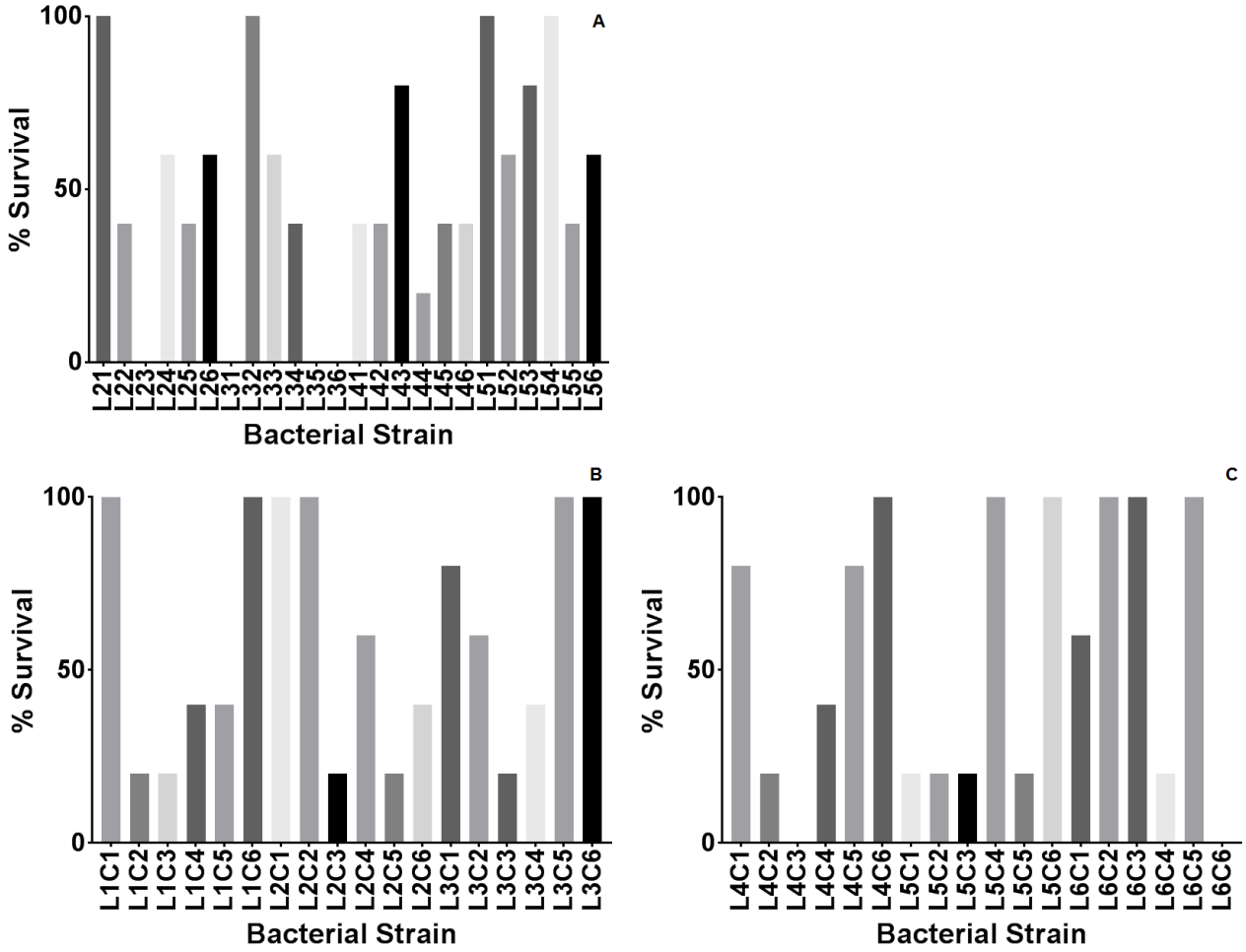


Figure 4.5: Survival of *G. mellonella* 1 h after injection with *P. aeruginosa* isolate supernatants. *C. elegans* evolved low host density (A) and low host density control (B and C) isolates pictured, 5 replicates per isolate.

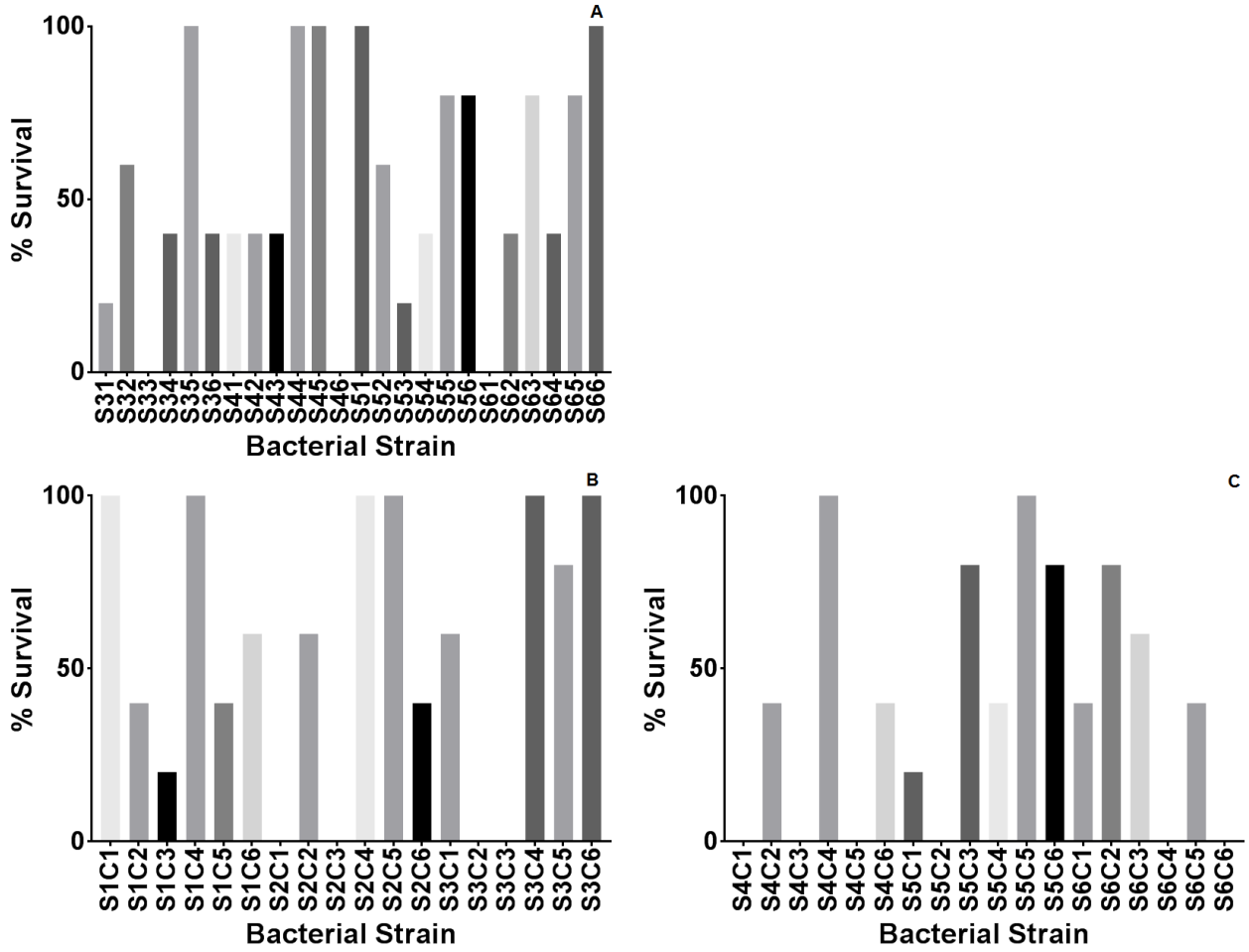


Figure 4.6: Survival of *G. mellonella* 1 h after injection with *P. aeruginosa* isolate supernatants. *C. elegans* evolved high host density (A) and high host density control (B and C) isolates pictured, 5 replicates per isolate.

A similar pattern to the low host density worm evolved group was shown by the high host density worm evolved isolates (Figure 4.6). Although only three isolates (S33, S46 and S61) displayed the maximum virulence, examples of both high and low virulence were present in every population group. A similar general pattern was present in the high host density control treatment group. Although the S1C and S6C populations were lacking one extreme of the virulence range, 0% and 100% survival respectively, both still contained examples of high and low virulence towards the *G. mellonella* larvae.

Mung bean assay growth is dependent on ambient environmental conditions

Despite identical experimental set ups, the three sets of mung bean assays performed in this study resulted in substantial differences in the growth of the negative controls (Figure 4.7). The only variables that could explain this variation are changes in environmental conditions, including varying laboratory temperature and external weather conditions, such as light levels, during the assay. These data highlight how varied the mung bean assay could be, with growth affected by external environmental factors. Therefore, each data set for this assay can only be reliably compared to the relevant negative control and not between each data set.

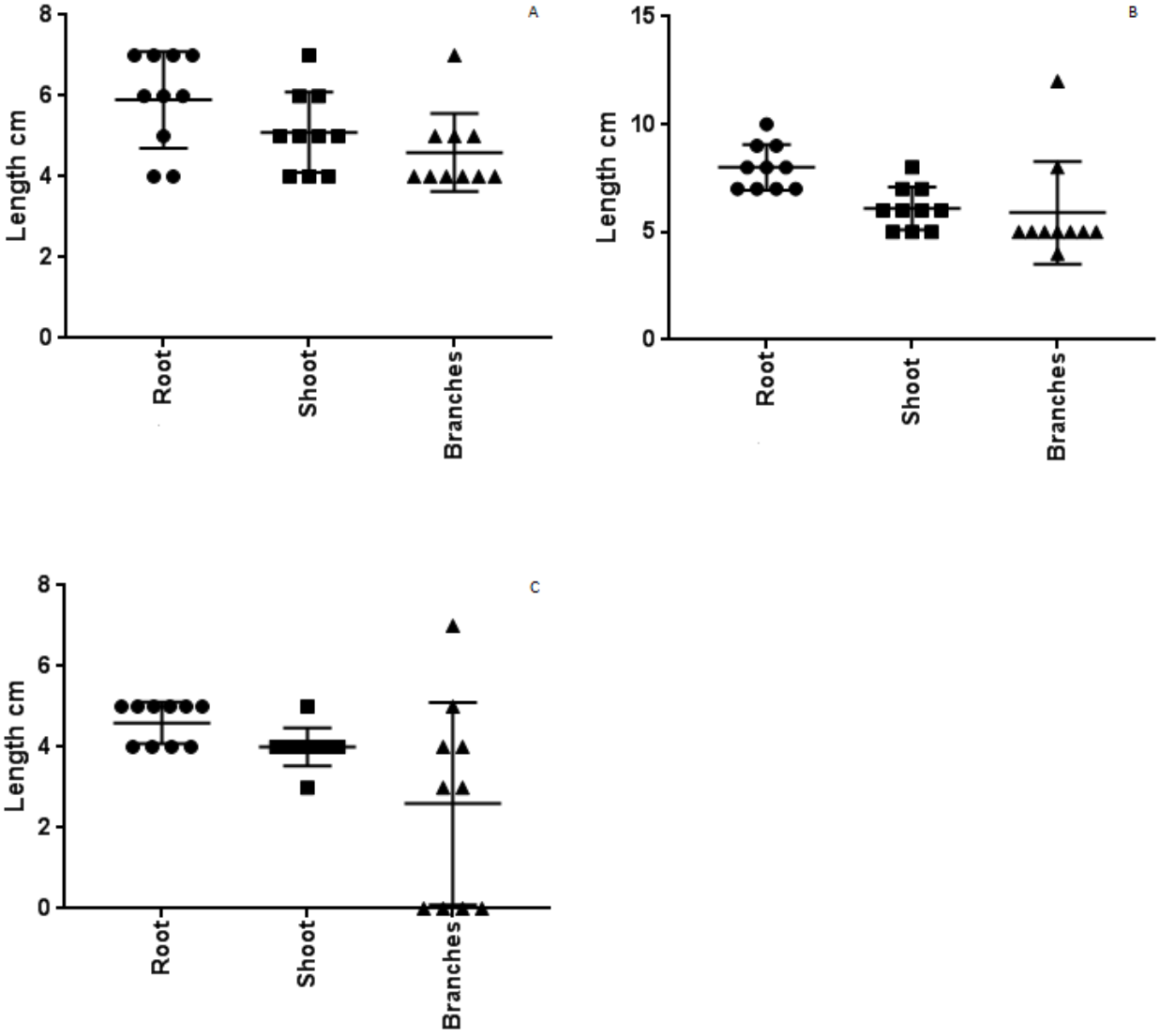


Figure 4.7: Growth of the negative controls for each of the 3 batches of mung bean assays performed. Despite all controllable experimental conditions being identical, the results were varied. Although the first two data sets (A and B) displayed similar levels of growth, the third-time point (C) displayed lower levels of growth.

P. aeruginosa does not negatively affect mung bean growth

Although a wide variety of strains and populations of *P. aeruginosa* were assayed for virulence, including laboratory strains, clinical isolates and *C. elegans* evolved populations and isolates from this study (see Chapter 3), little, if any signs of virulence were observed in the mung beans (Table 4.2), even the most virulent strain tested in the other hosts, wild type PA14, was not virulent in the beans (Figure 4.8). All beans, when grown with exposure to *P. aeruginosa*, did not deviate from the growth rates of the negative controls grown at the same time. Therefore, it appears that *P. aeruginosa* is not virulent towards *V. radiate*.

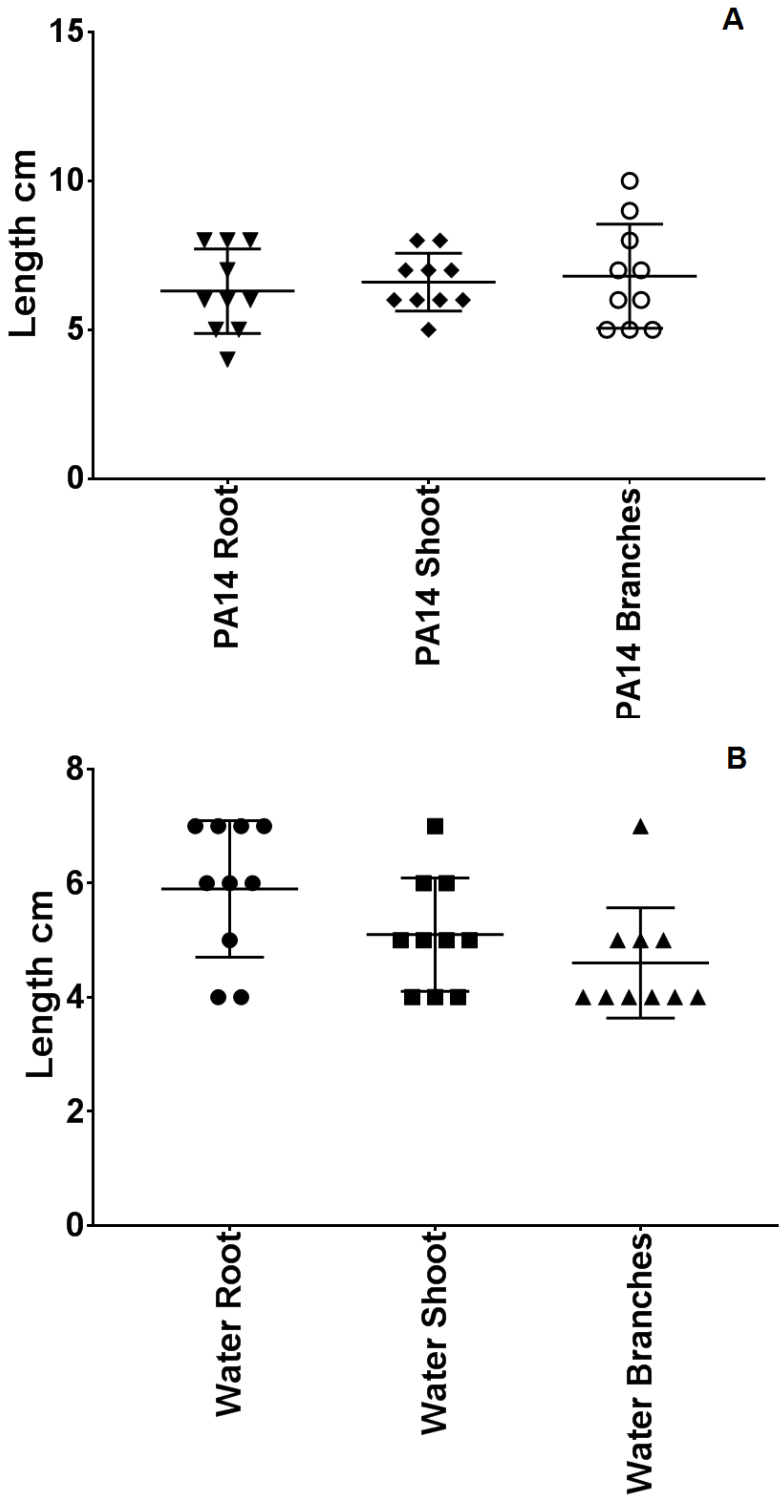


Figure 4.8: Mung bean growth in the presence of *P. aeruginosa* PA14 (A) and the water negative control performed at the same time (B). No attenuation of growth was observed in the presence of the bacteria. PA14 data is presented to highlight the lack of virulence *P. aeruginosa* displayed in the *V. radiate* model. Even the most virulent strain tested was avirulent in the mung bean model. All other tested strains displayed a similar lack of virulence (see Appendix 2 for full data sets).

Virulence of *P. aeruginosa* strains in different hosts summary

Bacteria	*C. elegans* Virulence	*G. mellonella* Virulence 1 h	*G. mellonella* Virulence 48 h	*V. radiate* Virulence
OP50	Low	Low	Low	None
PA14	High	High	High	None
PA14 *ΔlasR*	Low	Low	High	None
PA14 *ΔlasI*	Low	Intermediate	High	None
PA14 *ΔrhlR*	Low	Low	High	None
PA14 *ΔrhlI*	Low	Intermediate	High	None
PA14 *ΔpqsR*	Low	High	High	None
PA14 *ΔpqsE*	Low	High	High	None
PAO1-N	Low	High	High	None
PAO1-N *ΔpqsA*	Low	High	High	None
PAO1-N *ΔpqsE*	Low	Intermediate	High	None
PAO1-P	Low	Intermediate	High	None
PAO1-P *ΔpelA*	Low	High	High	None
PAO1-P *ΔpslBCD*	Low	Intermediate	High	None
PAO1-P *Δpel/psl*	Low	High	High	None
CW2 B1	Low	Low	High	None
CW3 B1	Low	Intermediate	High	None
CW3T1	Low	Intermediate	High	None
CW4 T1	Low	Intermediate	High	None
CW5 T1	Low	Low	High	None
CW5 T2	Low	High	High	None
SED2	Low	High	High	None
SED6	Low	Intermediate	High	None
SED8	Low	High	High	None
SED16	Low	High	High	None
SED20	Low	High	High	None
SED22	Low	High	High	None
AN1	High	Intermediate	High	None
L2	Low	Low	High	None
L3	Low	Low	High	None
L4	High	Intermediate	High	None

Bacteria	*C. elegans* Virulence	*G. mellonella* Virulence 1 h	*G. mellonella* Virulence 48 h	*V. radiate* Virulence
L5	Low	Low	High	None
L1C	High	High	High	None
L2C	Low	Intermediate	High	None
L3C	Low	Low	High	None
L4C	High	High	High	None
L5C	Low	Low	High	None
L6C	Low	Intermediate	High	None
S3	High	High	High	None
S4	High	Intermediate	High	None
S5	High	Intermediate	High	None
S6	High	High	High	None
S1C	High	High	High	None
S2C	High	Low	High	None
S3C	High	Intermediate	High	None
S4C	High	High	High	None
S5C	High	High	High	None
S6C	Low	Intermediate	High	None
L22	Low	Intermediate	High	None
L31	Low	High	High	None
L34	High	Intermediate	High	None
L41	High	Intermediate	High	None
L3C1	Low	Low	High	None
L3C2	Low	Intermediate	High	None
L6C3	High	Low	High	None
L6C6	Low	High	High	None
S51	Low	Low	High	None
S53	High	High	High	None
S61	High	High	High	None
S62	High	Intermediate	High	None
S1C1	High	Low	High	None
S6C1	Low	Intermediate	High	None
S6C4	High	High	High	None

Table 4.2: Summarised virulence data for *P. aeruginosa* in multiple hosts. *C. elegans* data taken from Chapter 3, low virulence defined as worm population reaching uncountable density, high virulence defined as worms being constrained to a countable population for the entire assay. For *G. mellonella* high virulence was defined as 80% mortality or higher, intermediate as between 20% and 80%, and low as 20% or less. For *V. radiate*, no virulence was recorded when the three tested growth parameters were consistent with that of the negative control grown at the same time. For full data sets of the *G. mellonella* and *V. radiate* assays, see Appendix 2.

Discussion

P. aeruginosa is considered to be an opportunistic pathogen with a wide spectrum of potential hosts. To observe how the virulence of *P. aeruginosa* can vary in different hosts, virulence was assayed in two organisms, waxmoth larvae (*G. mellonella*) and mung bean seedlings (*V. radiate*), to contrast with the work performed in *C. elegans* (Chapter 3).

Evolved *P. aeruginosa* populations and isolates (from Chapter 3) displayed host specific virulence phenotypes. While virulence in *C. elegans* varied substantially on a strain by strain basis (Chapters 2 and 3), the same was not true for both *G. mellonella* and *V. radiate*. *P. aeruginosa* was most virulent in the moth larvae, with all strains proving lethal, and least virulent in the bean seedlings, as no strain attenuated growth. *C. elegans* therefore, was of comparatively intermediate virulence, where variations in pathogen phenotype between strains play a greater role in pathogenicity than the host side of the relationship.

All *G. mellonella* larvae treated with *P. aeruginosa* supernatant died within 48 hours, regardless of the origin of the bacteria. Although the high lethality of *P. aeruginosa* in this host had been previously documented[225, 231], this work has been done using inoculums of live bacteria. This study, on the other hand, achieved similar results using supernatants. *P. aeruginosa* was never in direct contact with the larvae, yet this was still enough to induce death, even with the least virulent of isolates. Unfortunately, the extreme virulence of *P. aeruginosa* in this host organism presents issues in its further use in the study of long term virulence. While not necessarily impossible, it would be difficult to perform a

long-term selection experiment using this model. Just like with the fast kill conditions for *C. elegans*, the high virulence would necessitate an extremely short incubation time in host before passage. It would also increase the likelihood of losing particularly virulent strains from evolved populations, as they would kill their hosts even quicker, and potentially be lost from the population along with the dead hosts**.** Overall, in spite of their recent popularity as a model organism, *G. mellonella* larvae appear to be far from ideal for the study of *P. aeruginosa* virulence evolution. While it will doubtless continue to find use in testing new therapies[231] and antimicrobials[239], and as a cheap precursor to fine tune mammalian studies[200], *P. aeruginosa* is simply too lethal for evolutionary selection studies using *G. mellonella* larvae.

P. aeruginosa is far more virulent in *G. mellonella* larvae than it is in humans (although *P. aeruginosa* can of course be extremely dangerous to humans in certain circumstances, such as burn wounds[240]). One must then consider the level of variation seen in killing times. While some studies report wild type strains of *P. aeruginosa* killing larvae within 24 h of initial exposure[226, 227, 239], others have reported killing after 48 h[200, 231]. Interestingly, in these studies, PAO1 was apparently lethal to the larvae faster than PA14, despite being considered a less virulent strain of *P. aeruginosa* (a conclusion supported by this study). Although the wide variety of PAO1 strains in use worldwide is a possible explanation for the anomalously high virulence previously reported, it is not the only explanation. As a relatively new model, *G. mellonella* lacks a lot of the established, standardised protocols seen in more mature model organisms. Most

striking is a lack of 'laboratory strains' for use in studies, there are no stock centres for this organism and samples are obtained via commercial pet food suppliers[197]. So, the variations in lethality seen in different studies may be due to undetected variation in their supply of host organisms. The inherent variation in this model does raise issues regarding the clinical relevance of any studies performed in this host organism. Can results reliably be extrapolated to human victims of *P. aeruginosa* infection, when there is such discrepancy between insect studies?

However, if the moth larvae were examined early enough after inoculation, at the 1 h time point, then some variation in virulence could be observed. Both host densities readily produced examples of high, intermediate and low *G. mellonella* virulence. Therefore *C. elegans* host density does not appear to be an important evolutionary pressure on *P. aeruginosa* virulence in this alternate host model. However, the same variety of phenotypes was also seen in the control treatment groups, meaning that the presence of the *C. elegans* host is ultimately irrelevant in the evolution of virulence towards *G. mellonella*. The lack of difference in worm evolved and control treatment groups suggests that evolution in a *C. elegans* host does not influence the evolution of *P. aeruginosa* virulence towards *G. mellonella* in any particular direction. This may simply be due to the difference in *P. aeruginosa* lethality in the two model organisms. As the bacteria is notably less virulent in *C. elegans* compared with *G. mellonella*, even with all its virulence machinery intact, it is possible that the *C. elegans* host environment will only degrade virulence to the point that the host can endure the infection, but this

attenuated virulence level could still be dangerous to the less hardy *G. mellonella* larvae. It is also possible that different virulence pathways are at work in the two model organisms. Although it has been claimed that protease activity is key to *P. aeruginosa* virulence towards *G. mellonella*[231], several isolates produced by this study, including S61, S3C4, L23 and L6C6, were negative for protease activity (see Chapter 3), yet retained high virulence in *G. mellonella*. This suggests that *P. aeruginosa* can utilise a substantial proportion of its diverse virulence machinery in *G. mellonella*, and may in fact utilise virulence factors that are not important in the *C. elegans* host. If true, this would readily explain the variations seen in this study, as unused virulence pathways would be unlikely to be affected by evolutionary pressures of an environment in which they remain unused. Therefore, despite the overall high virulence of *P. aeruginosa* in this host, the 1 h after inoculation time point could be used to test potential differences in virulence between strains.

Previous work suggested that the mung bean assay was a viable model for studying *P. aeruginosa* virulence (Garge, Unpublished data), with the presence of virulent *P. aeruginosa* impairing the growth of the bean seedlings. However, the results of this study suggest the opposite. No *P. aeruginosa* strain, population or isolate tested caused any noteworthy attenuation of mung bean growth. What little growth attenuation occurred was limited to a single growth condition, root length, and was so small compared to the negative controls that it was most likely an experimental artefact.

It was the hope of this study that the mung bean assay would prove to be another viable plant model in which to study *P. aeruginosa*, to complement the several that already exist. However, unlike the established models for *Arabidopsis thaliana* [182, 232-234], basil[233] and lettuce[146] which are susceptible to *P. aeruginosa* infection, in this study mung beans were not. The unavoidable conclusion of this set of assays is that *P. aeruginosa* has no appreciable effect on the growth of mung bean seedlings, and therefore that this host organism is not a suitable model for further study.

While virulence could be highly varied between different *P. aeruginosa* strains in the nematode (as seen Chapters 2&3), the two hosts tested here were far more uniform. *G. mellonella* appears to be a host in which *P. aeruginosa* is always highly virulent and deadly. However, the opposite was the case for *V. radiate*. As *P. aeruginosa* infection had no discernible effect on the growth of the beans, it would appear that *P. aeruginosa* is not virulent in this host. The high degree of variation seen between these three tested host organisms serves to support the idea that virulence as a property is not solely the domain of the microbe, but rather the result of a unique combination of microbe and host interactions[9]. Before this experiment, we hypothesised that at least some signs of virulence would occur in the mung bean, especially given the results of previous experiments (Garge, Unpublished data). However this complete defiance of our predictions is in keeping with the idea that, thanks to the volume of potential host-microbe interactions, virulence is extremely difficult, if not impossible, to predict, especially in the case of a novel host and microbe interaction[7].

There are two possibilities to explain the lack of virulence in *V. radiate.* The host may simply not be susceptible to pathogenesis by *P. aeruginosa*, the behaviours which cause disease in other organisms simply do not do so in *V. radiate*. Alternatively, avirulence may be the result of phenotypic plasticity, where the same genotype can produce different phenotypes in different environmental circumstances, so certain virulence pathways are not activated when *P. aeruginosa* encounters *V. radiate*. Of these two options, phenotypic plasticity is the more likely explanation, as *P. aeruginosa* possesses a complex and variable set of regulatory machinery[241]. Given the variety of environments *P. aeruginosa* encounters, the advantage of a plastic phenotype outweighs the resource cost of maintaining the required regulatory machinery. The lack of virulence in *V. radiate* presents the possibility that *P. aeruginosa* is capable of utilising some host organisms as reservoirs and vectors for infection, in addition to its presence environmentally, outside of a host. This result highlights the varied nature of an opportunistic pathogen. Although, as generalists, they can and do cause disease in a variety of host organisms, pathogenicity is not inevitable or universal. Just like a specialist pathogen encountering the wrong host, environmental opportunists, such as *P. aeruginosa*, will be benign towards certain organisms.

Alternatively, the difference in virulence observed could be the result of the differing infection routes required by the different assays, oral infection for *C. elegans*, injection for *G. mellonella* and external exposure for *V. radiate*. Of these three methods, the most invasive, direct injection, produced the most virulent

outcome, while the least invasive, external exposure, resulted in the lowest virulence. While this could be taken to indicate that method of infection plays an equal, or more important, role in the emergence of virulence than the specifics of the host organism, the reality is somewhat more complex. Differentiating between infection route and host organism often proves impossible, as different infection routes will not work equally in all host organisms. For example, infecting *C. elegans* via injection would be an extremely difficult process, due to the size and durability of the organism, with injection itself likely proving fatal. On the other hand, infection route may just not be as important as one might assume. During our attempts to refine the *V. radiate* assay, direct injection of live bacteria was attempted as an alternative infection method to external exposure, with the same lack of growth attenuation (data not shown). This similarity of virulence outcome across two different infection routes for *V. radiate* lends credence to the idea that the nature of the host organism is the greater determinant in virulence outcome than infection route.

In conclusion, both alternate host organisms tested did display different virulence phenotypes to that of *C. elegans,* meaning that any evolved change in *P. aeruginosa* is the result of host specific adaption rather than a generalist 'host adaption'. At that 1 h time point, all evolved population groups displayed a full range of virulence phenotypes, unlike in *C. elegans,* where there was a greater similarity in phenotypes between isolates from the same population. However, *P. aeruginosa* was ultimately far more virulent in the *G. mellonella* model, with variations in virulence only visible when a very short assay timescale was used,

otherwise all strains produced rapid larvae death. The mung bean model was the opposite; *P. aeruginosa* proved to be ultimately irrelevant to bean growth, regardless of the origin of the bacteria used. These assays highlight the degree of variation between hosts virulence can display. Once again, these data highlight the vital importance of choosing the correct model organism when designing a study, given the wide variety in virulence phenotypes identified here. For example, neither of the alternate hosts tested in this chapter would have been have made good alternatives to *C. elegans* in the previously performed selection experiment. Virulence of *P. aeruginosa* is therefore highly dependent on the nature of the host organism.

Chapter 5: General Discussion

Summary

The understanding of virulence, and the conditions under which it can arise, is fundamental if we are to continue expanding our knowledge of disease causing bacteria, especially in an age of increasing antibiotic resistance[242]. If the circumstances under which virulence emerges and increases can be better understood, there is potential for holistic treatment approaches to minimise the chances of highly virulent strains emerging, as well as focussing future research efforts on the causes of pathogenicity. However, this lofty ideal will require a substantial understanding of the realities of virulence evolution if sufficiently robust and helpful predictive models are to be designed.

This study examined the both evolution of *P. aeruginosa* virulence, and how that virulence changes depending on the bacterial strain and host organism. In the *C. elegans* host virulence varies widely, from the highly virulent PA14 to essentially avirulent strains containing specific mutations. However, this varied with strain, as PAO1 strains universally proved to be less virulent than PA14 (Chapter 2). The most striking outcome was the similarities between laboratory strains and clinical isolates, with all tested cystic fibrosis and chronic wound isolates bearing more similarities to the less virulent PAO1 than PA14. This was in keeping with the clinical origins of the respective *P. aeruginosa* strains and highlighted the effect different environments can have on virulence levels.

The results of the selection experiment in Chapter 3 show the high level of variability evolving bacteria can display, with both high and low virulence strains evolved. Unfortunately, when controls were accounted for, there was little difference between *P. aeruginosa* evolved in a low host density environment compared with a high host density environment. Therefore, the initial hypothesis of the selection experiment (that bacteria evolved in high host density conditions would retain virulence more than those evolved in low host density conditions) could not be confirmed. These results highlight the divergent phenotypes that can occur on Petri dishes, emphasising the need to keep working strains regularly refreshed from frozen stocks, rather than repeatedly sub culturing bacteria. Although levels of virulence factors did vary, a combination of several did correlate with *C. elegans* killing, suggesting virulence in this host organism is a property of multiple virulence factors. However, there was no clear genetic basis for these phenotypes observed in the sequencing data.

When *P. aeruginosa* virulence was examined in alternate host organisms (Chapter 4), the nature of the host organism played a greater role in the virulence profile than the inherent virulence profile of the bacteria. While virulence did vary in *C. elegans*, it was always high (with some variation) in *G. mellonella*, while no tested strain or population had any notable virulence in *V. radiate*. Chapter 2 showed that different *P. aeruginosa* strains displayed different virulence levels which was dependent on their environment of origin, and therefore demonstrated the need for careful strain choice during study design. In contrast the Chapter 4 results offer the other side of the host-pathogen

relationship, where virulence varies substantially by host organism, therefore emphasising the need for careful selection of host model when designing virulence studies.

The Effect of Host Density on Virulence Evolution

The central hypothesis that the selection experiment (Chapter 3) set out to answer was "can *P. aeruginosa* virulence be attenuated in a low host density environment?", so that the environment would favour the attenuation of virulence for longer time in host, in accordance with the trade-off hypothesis[18, 19, 31, 35]. Unfortunately, the greatest difference amongst the evolved *P. aeruginosa* was between the two worm evolved cohorts and the controls, rather than between the high and low host density worm evolved cohorts. So, the presence of the *C. elegans* host did not exert any major evolutionary pressures in the virulence of *P. aeruginosa*. This result runs contrary to a recent study[174], which reported the consistent emergence of *lasR* mutations and virulence attenuation in the *C. elegans* host. However, the lack of controls in the previous study does undermine their conclusions, especially in light of the findings of this selection experiment. The question then becomes why this was the case.

The issue may be the ultimate nature of the chosen pathogen, *P. aeruginosa*. Unlike some other pathogenic organisms, *P. aeruginosa* is an opportunist, and is capable of existing independently of a host organism in the wider environment. Opportunistic pathogens have already been identified as presenting unique issues in the study of virulence, compared with primary pathogens[79]. It has been suggested that fluctuating between in-host and outside-host environments

promotes lowered virulence by preventing extreme specialisation[49]. It may follow that being able to survive outside the host for extended periods disrupts the trade off equilibrium. However, this disruption could occur in either direction. If the pathogen is not fully dependent on the host, then it stands to lose less by killing the host, so there could be few selection pressures acting against heightened or retained virulence. On the other hand, if the pathogen is not reliant on the host to propagate and spread, then the increased transmissibility gained from higher virulence levels may not provide any competitive advantage, as the pathogen can exist just as readily outside of a host, and therefore one might expect to find a lower virulence phenotype, as the pathogen gains a larger advantage from a decreased metabolic load. Put another way, *P. aeruginosa* doesn't require the presence of *C. elegans,* which appears to have limited the amount of evolutionary variation produced by the selection experiment.

Virulence variation across hosts

There were readily apparent differences in the behaviour of *P. aeruginosa* when placed in different host organisms during this study. Levels of virulence towards *C. elegans* showed substantial differences depending on strain or population, from highly virulent to indistinguishable from the avirulent OP50 stock food source (Chapters 2 and 3). This contrasted with the data from the alternate hosts *G. mellonella* and *V. radiate* (Chapter 5), both of which were far more uniform. Despite some variation between strains used, *P. aeruginosa* exposure always resulted in rapid death for the moth larvae, while no attenuation of growth was

observed for the mung beans, regardless of the type of *P. aeruginosa* used. Taken as a whole, the data from these 3 host organisms demonstrates that virulence should never be assumed, that *P. aeruginosa* will always be more dangerous to some organisms than other, regardless of its evolutionary history or pathogenicity in a different organism. This is consistent with the idea that the function of the host plays just an equally important role to that of the pathogen in the manifestation of disease[7-9].

The impact of QS on *P. aeruginosa* virulence

The QS systems of *P. aeruginosa* have long been implicated in the virulence of the bacteria[147, 192, 193]. This finding was replicated in Chapter 2, with the tested PA14 QS mutants all displaying attenuated virulence compared with the wildtype PA14. These results were consistent with published findings that QS genes are a major controller of *C. elegans* pathogenicity and virulence[243]. However, this importance of individual QS components in *P. aeruginosa* virulence was somewhat undermined by the sequencing data from Chapter 3, which did not contain any non-silent mutations in any of the QS genes. This finding is more in keeping with a recent study challenging the simple hierarchical view of *P. aeruginosa* quorum sensing[16], which put forward the hypothesis that established hierarchical view of QS is overly simplistic. Under this explanation, *P. aeruginosa* is able to access the benefits normally associated with particular QS signals via alternate strategies. If true, then loss of function mutations in specific *P. aeruginosa* QS genes are less important than has previously been assumed. This would be in keeping with this study's sequencing

data, which showed that evolved mutants do not require mutations in the key QS genes to evolve attenuated virulence towards *C. elegans*.

Another possible explanation for the lack of obvious QS mutants in the evolved populations may be due to their ancestor strain, PA14. Naturally occurring QS mutants, while common in chronic infections, have been reported as being much more infrequent in acute infections[16]. As PA14 was initially derived from an acute burn wound[146], it is possible that the strain is predisposed to resist loss of function mutations in QS genes. In the bacteria's original environment, there would be a selection pressure against highly mutable QS genes, and this resistance could have been preserved into the laboratory strain PA14, along with the heightened virulence.

Future Work

The virulence of laboratory strains of *P. aeruginosa* varied according to their clinical origin, which chronic infections producing less virulent strains (Chapter 2). While this pattern was replicated for the clinical isolates tested, expanding that experiment to include fresh clinical isolates from acute *P. aeruginosa* infections, such as burn wounds, would be a worthwhile extension of this study. This would better substantiate that it is the environment of the clinical context that establishes the virulence level seen in the laboratory strains, and that the high virulence of PA14 is not an artefact of it spending 20 years *in vitro* as a laboratory organism.

Although some genomic data was obtained for the evolved populations produced by the selection experiment (Chapter 3), this avenue of analysis could benefit from additional scrutiny, especially as the sequencing data presented here did not reveal any especially informative genes implicated in *P. aeruginosa* virulence. The sequencing data obtained here was limited only to the presence of SNPs at the population level. The frequency with which these SNPs appeared within the populations could not be determined. More detail at this level would enable a more accurate assessment of how likely the present functional SNPs are to effect *P. aeruginosa* virulence. Additionally, sequencing some of the individual evolved isolates would offer additional opportunities to identify the key genes at work in *C. elegans* virulence. This would be especially true for isolates that displayed very different virulence phenotypes, despite undergoing the same experimental conditions, for example the L34 isolate was uniquely virulent among the isolates taken from that evolved.

The testing of evolved *P. aeruginosa* in alternative host organisms could also be expanded. Other potential hosts to test include plant models such as *Arabidopsis thaliana*[182], fruit flies[244] and live mammal models such as mice[245]. Individual tissue models, such as a recently developed *ex vivo* pig lung culture[246] could also be tested, although some alterations here would be required, as severity of virulence, measured by host death or growth attenuation in this study, could not be applied to separated tissue types. While this study only tested *P. aeruginosa* virulence in 3 hosts, drastic variations in virulence were observed (Chapter 4). Adding to this repertoire of host organisms would allow

for a more accurate assessment of the relative advantages of each host regarding *P. aeruginosa* virulence under specific environmental conditions, such as the differing environments of acute and chronic infections. Coupled with the differences in virulence between *P. aeruginosa* strains of varying clinical origin (Chapter 2), this could allow for a more customised methodology for the study of *P. aeruginosa* virulence, where both host organism and bacteria can be carefully chosen to closely match the clinical circumstance of interest.

In conclusion, the results presented in this thesis highlight the varied nature of host-pathogen interactions and the inherent variability of the evolutionary process. The findings suggest that *P. aeruginosa* virulence is highly dependent on the environment of origin. This finding highlights the importance of careful choice of strain when designing studies, as acute and chronic strains have the potential to produce extremely different phenotypes. *P. aeruginosa* virulence was also shown to be a multifactoral process, requiring the action of several virulence factors to induce killing of *C. elegans*, a finding consistent with that already reported[5]. Another important factor in *P. aeruginosa* virulence proved to be choice of host organism, the 3 hosts tested here all displayed very different susceptibilities to *P. aeruginosa* infection. This reinforces the idea of virulence being the product of host-microbe interactions, rather than solely the purview of the microbe[7], and emphasises the need to choose the appropriate host organism when designing future studies to avoid unrepresentative results. Additionally, the results of the selection experiment run contrary to the reported development of commensalism, and the importance of *lasR* in the virulence of *P.*

aeruginosa towards *C. elegans*[174]. The previously published study did not include controls in its selection experiment, whereas this study observed similar phenotypes emerging in both worm evolved and control *P. aeruginosa*. Therefore, it would appear that some attenuation of *P. aeruginosa* virulence should be expected as a result of extended periods of subculture, regardless of the presence of *C. elegans*.

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Appendix 1: *P. aeruginosa* evolved strain list and phenotype data

Strain	Description	Source
Populations		
L2	Low Host Density *P. aeruginosa* population 2, evolved from AN1.	This Study
L3	Low Host Density *P. aeruginosa* population 3, evolved from AN1.	This Study
L4	Low Host Density *P. aeruginosa* population 4, evolved from AN1.	This Study
L5	Low Host Density *P. aeruginosa* population 5, evolved from AN1.	This Study
L1C	Low Host Density *P. aeruginosa* control population 1, evolved from AN1.	This Study
L2C	Low Host Density *P. aeruginosa* control population 2, evolved from AN1.	This Study
L3C	Low Host Density *P. aeruginosa* control population 3, evolved from AN1.	This Study
L4C	Low Host Density *P. aeruginosa* control population 4, evolved from AN1.	This Study
L5C	Low Host Density *P. aeruginosa* control population 5, evolved from AN1.	This Study
L6C	Low Host Density *P. aeruginosa* control population 6, evolved from AN1.	This Study
S3	High Host Density P. *aeruginosa* population 3, evolved from AN1.	This Study
S4	High Host Density *P. aeruginosa* population 4, evolved from AN1.	This Study
S5	High Host Density *P. aeruginosa* population 5, evolved from AN1.	This Study
S6	High Host Density *P. aeruginosa* population 6, evolved from AN1.	This Study
S1C	High Host Density *P. aeruginosa* control population 1, evolved from AN1.	This Study
S2C	High Host Density *P. aeruginosa* control population 2, evolved from AN1.	This Study
S3C	High Host Density *P. aeruginosa* control population 3, evolved from AN1.	This Study
S4C	High Host Density *P. aeruginosa* control population 4, evolved from AN1.	This Study

Strain	Description	Source
S5C	High Host Density *P. aeruginosa* control population 5, evolved from AN1.	This Study
S6C	High Host Density *P. aeruginosa* control population 6, evolved from AN1.	This Study
Individual Isolates		
L21	First Isolate from L2 population.	This Study
L22	Second Isolate from L2 population.	This Study
L23	Third Isolate from L2 population.	This Study
L24	Fourth Isolate from L2 population.	This Study
L25	Fifth Isolate from L2 population.	This Study
L26	Sixth Isolate from L2 population.	This Study
L31	First Isolate from L3 population.	This Study
L32	Second Isolate from L3 population.	This Study
L33	Third Isolate from L3 population.	This Study
L34	Fourth Isolate from L3 population.	This Study
L35	Fifth Isolate from L3 population.	This Study
L36	Sixth Isolate from L3 population.	This Study
L41	First Isolate from L4 population.	This Study
L42	Second Isolate from L4 population.	This Study
L43	Third Isolate from L4 population.	This Study
L44	Fourth Isolate from L4 population.	This Study
L45	Fifth Isolate from L4 population.	This Study
L46	Sixth Isolate from L4 population.	This Study
L51	First Isolate from L5 population.	This Study
L52	Second Isolate from L5 population.	This Study
L53	Third Isolate from L5 population.	This Study
L54	Fourth Isolate from L5 population.	This Study
L55	Fifth Isolate from L5 population.	This Study
L56	Sixth Isolate from L5 population.	This Study
L1C1	First Isolate from L1C population.	This Study

Strain	Description	Source
L1C2	Second Isolate from L1C population.	This Study
L1C3	Third Isolate from L1C population.	This Study
L1C4	Fourth Isolate from L1C population.	This Study
L1C5	Fifth Isolate from L1C population.	This Study
L1C6	Sixth Isolate from L1C population.	This Study
L2C1	First Isolate from L2C population.	This Study
L2C2	Second Isolate from L2C population.	This Study
L2C3	Third Isolate from L2C population.	This Study
L2C4	Fourth Isolate from L2C population.	This Study
L2C5	Fifth Isolate from L2C population.	This Study
L2C6	Sixth Isolate from L2C population.	This Study
L3C1	First Isolate from L3C population.	This Study
L3C2	Second Isolate from L3C population.	This Study
L3C3	Third Isolate from L3C population.	This Study
L3C4	Fourth Isolate from L3C population.	This Study
L3C5	Fifth Isolate from L3C population.	This Study
L3C6	Sixth Isolate from L3C population.	This Study
L4C1	First Isolate from L4C population.	This Study
L4C2	Second Isolate from L4C population.	This Study
L4C3	Third Isolate from L4C population.	This Study
L4C4	Fourth Isolate from L4C population.	This Study
L4C5	Fifth Isolate from L4C population.	This Study
L4C6	Sixth Isolate from L4C population.	This Study
L5C1	First Isolate from L5C population.	This Study
L5C2	Second Isolate from L5C population.	This Study
L5C3	Third Isolate from L5C population.	This Study
L5C4	Fourth Isolate from L5C population.	This Study
L5C5	Fifth Isolate from L5C population.	This Study
L5C6	Sixth Isolate from L5C population.	This Study

Strain	Description	Source
L6C1	First Isolate from L6C population.	This Study
L6C2	Second Isolate from L6C population.	This Study
L6C3	Third Isolate from L6C population.	This Study
L6C4	Fourth Isolate from L6C population.	This Study
L6C5	Fifth Isolate from L6C population.	This Study
L6C6	Sixth Isolate from L6C population.	This Study
S31	First Isolate from S3 population.	This Study
S32	Second Isolate from S3 population.	This Study
S33	Third Isolate from S3 population.	This Study
S34	Fourth Isolate from S3 population.	This Study
S35	Fifth Isolate from S3 population.	This Study
S36	Sixth Isolate from S3 population.	This Study
S41	First Isolate from S4 population.	This Study
S42	Second Isolate from S4 population.	This Study
S43	Third Isolate from S4 population.	This Study
S44	Fourth Isolate from S4 population.	This Study
S45	Fifth Isolate from S4 population.	This Study
S46	Sixth Isolate from S4 population.	This Study
S51	First Isolate from S5 population.	This Study
S52	Second Isolate from S5 population.	This Study
S53	Third Isolate from S5 population.	This Study
S54	Fourth Isolate from S5 population.	This Study
S55	Fifth Isolate from S5 population.	This Study
S56	Sixth Isolate from S5 population.	This Study
S61	First Isolate from S6 population.	This Study
S62	Second Isolate from S6 population.	This Study
S63	Third Isolate from S6 population.	This Study
S64	Fourth Isolate from S6 population.	This Study
S65	Fifth Isolate from S6 population.	This Study

Strain	Description	Source
S66	Sixth Isolate from S6 population.	This Study
S1C1	First Isolate from S1C population.	This Study
S1C2	Second Isolate from S1C population.	This Study
S1C3	Third Isolate from S1C population.	This Study
S1C4	Fourth Isolate from S1C population.	This Study
S1C5	Fifth Isolate from S1C population.	This Study
S1C6	Sixth Isolate from S1C population.	This Study
S2C1	First Isolate from S2C population.	This Study
S2C2	Second Isolate from S2C population.	This Study
S2C3	Third Isolate from S2C population.	This Study
S2C4	Fourth Isolate from S2C population.	This Study
S2C5	Fifth Isolate from S2C population.	This Study
S2C6	Sixth Isolate from S2C population.	This Study
S3C1	First Isolate from S3C population.	This Study
S3C2	Second Isolate from S3C population.	This Study
S3C3	Third Isolate from S3C population.	This Study
S3C4	Fourth Isolate from S3C population.	This Study
S3C5	Fifth Isolate from S3C population.	This Study
S3C6	Sixth Isolate from S3C population.	This Study
S4C1	First Isolate from S4C population.	This Study
S4C2	Second Isolate from S4C population.	This Study
S4C3	Third Isolate from S4C population.	This Study
S4C4	Fourth Isolate from S4C population.	This Study
S4C5	Fifth Isolate from S4C population.	This Study
S4C6	Sixth Isolate from S4C population.	This Study
S5C1	First Isolate from S5C population.	This Study
S5C2	Second Isolate from S5C population.	This Study
S5C3	Third Isolate from S5C population.	This Study
S5C4	Forth Isolate from S5C population.	This Study

Strain	Description	Source
S5C5	Fifth Isolate from S5C population.	This Study
S5C6	Sixth Isolate from S5C population.	This Study
S6C1	First Isolate from S6C population.	This Study
S6C2	Second Isolate from S5C population.	This Study
S6C3	Third Isolate from S5C population.	This Study
S6C4	Fourth Isolate from S5C population.	This Study
S6C5	Fifth Isolate from S5C population.	This Study
S6C6	Sixth Isolate from S5C population.	This Study

Table X1.1: Summary of evolved *P. aeruginosa* populations and isolates produced during this study.

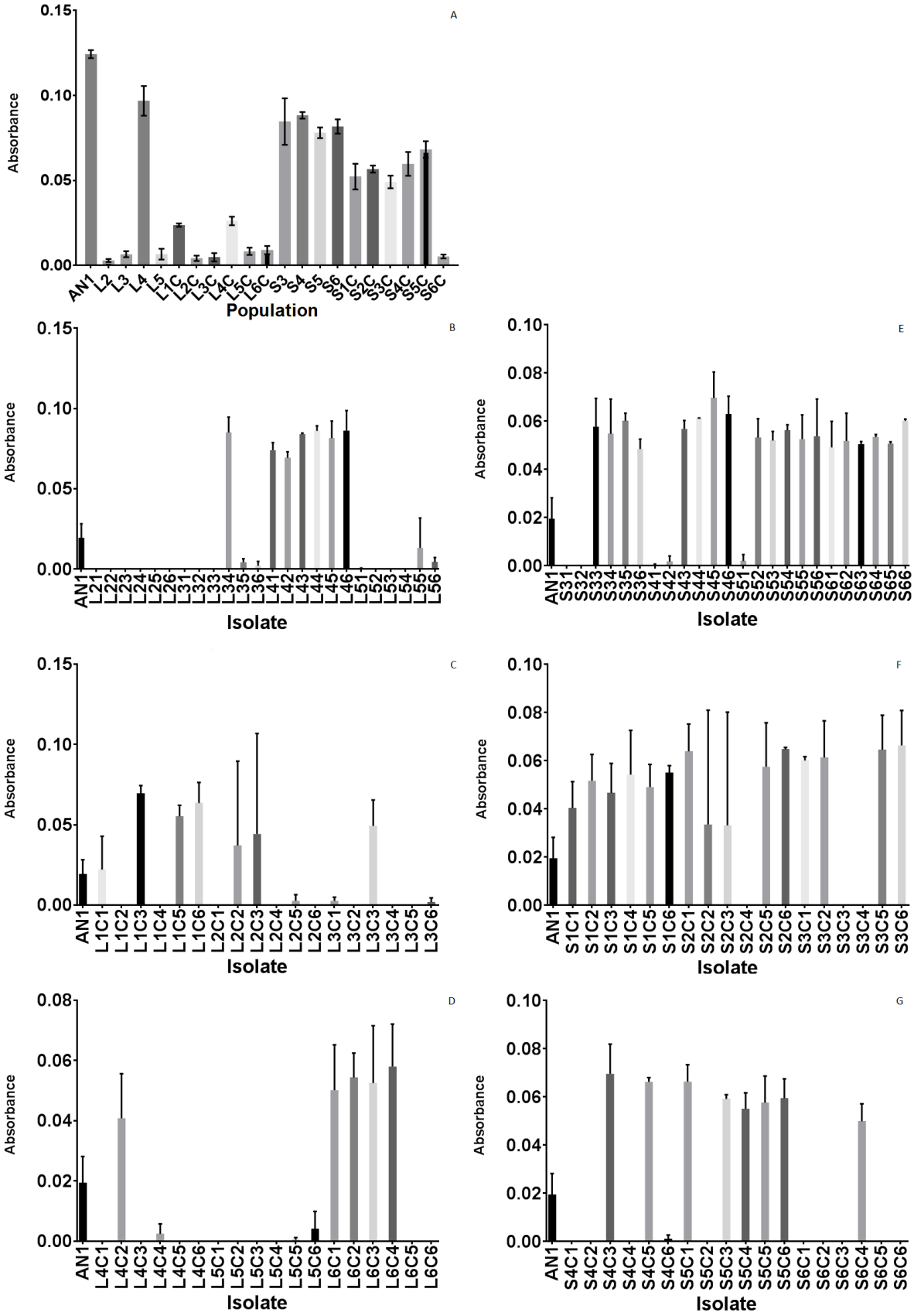


Figure X1.1: Pyocyanin output for all tested bacteria. Evolved populations (A) low host density worm evolved (B), low host density controls (C and D), high host density worm evolved (E) and high host density controls (F and G). Each bar represents the mean of a pair of readings.

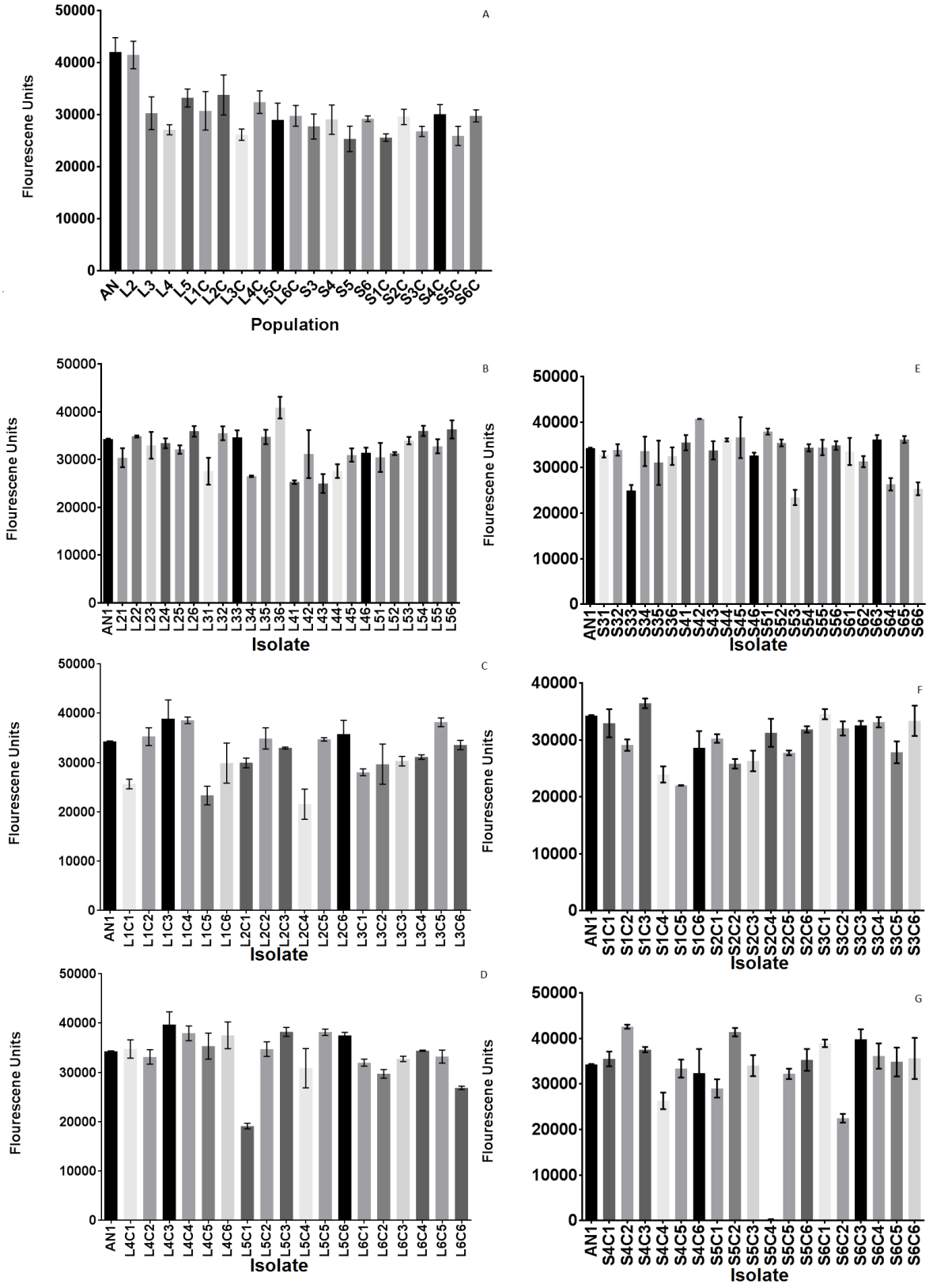


Figure X1.2: Pyoverdin output for all tested bacteria. Evolved populations (A) low host density worm evolved (B), low host density controls (C and D), high host density worm evolved (E) and high host density controls (F and G). Each bar represents the mean of three readings.

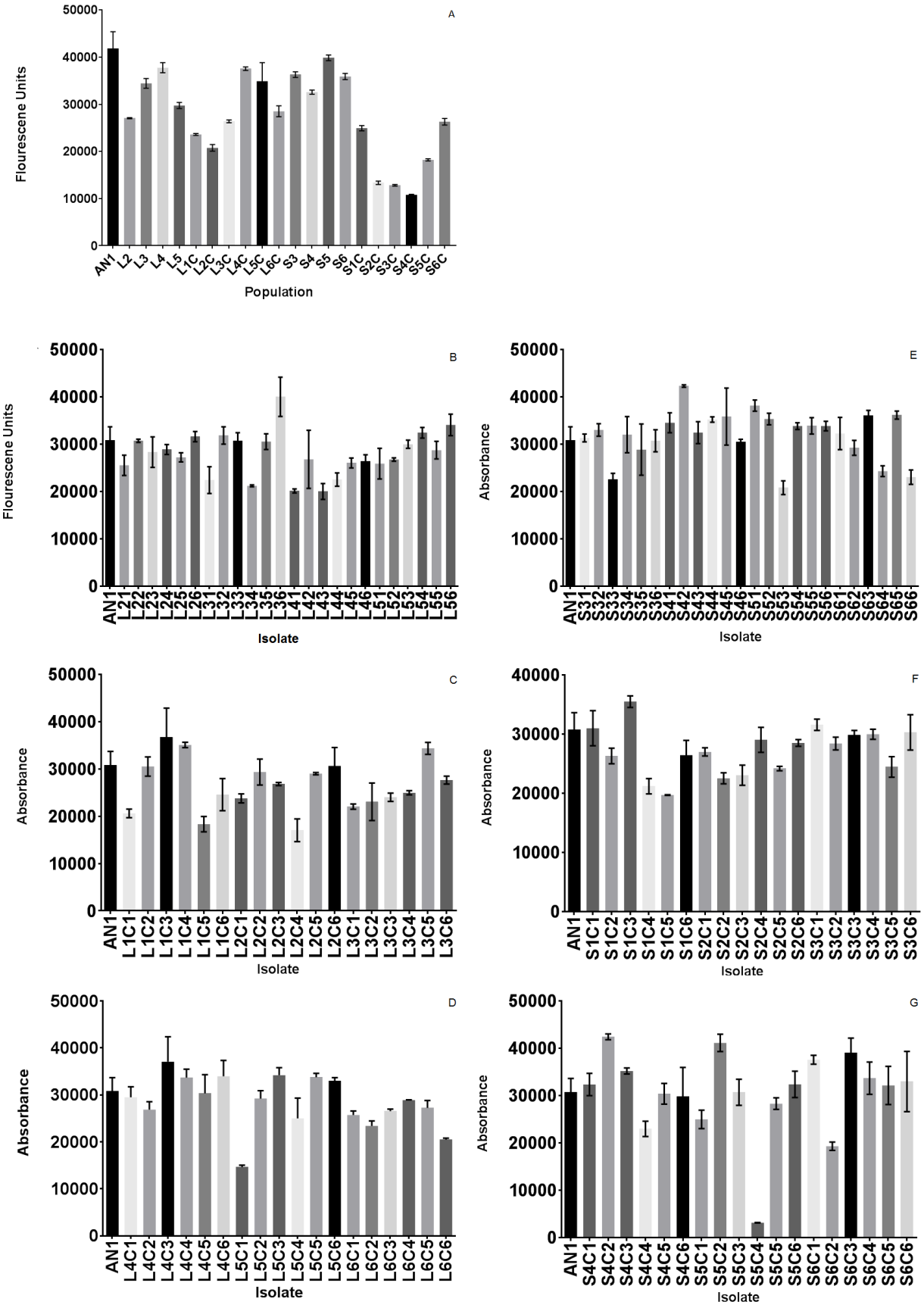


Figure X1.3: Pyochelin output for all tested bacteria. Evolved populations (A) low host density worm evolved (B), low host density controls (C and D), high host density worm evolved (E) and high host density controls (F and G). Each bar represents the mean of three readings.

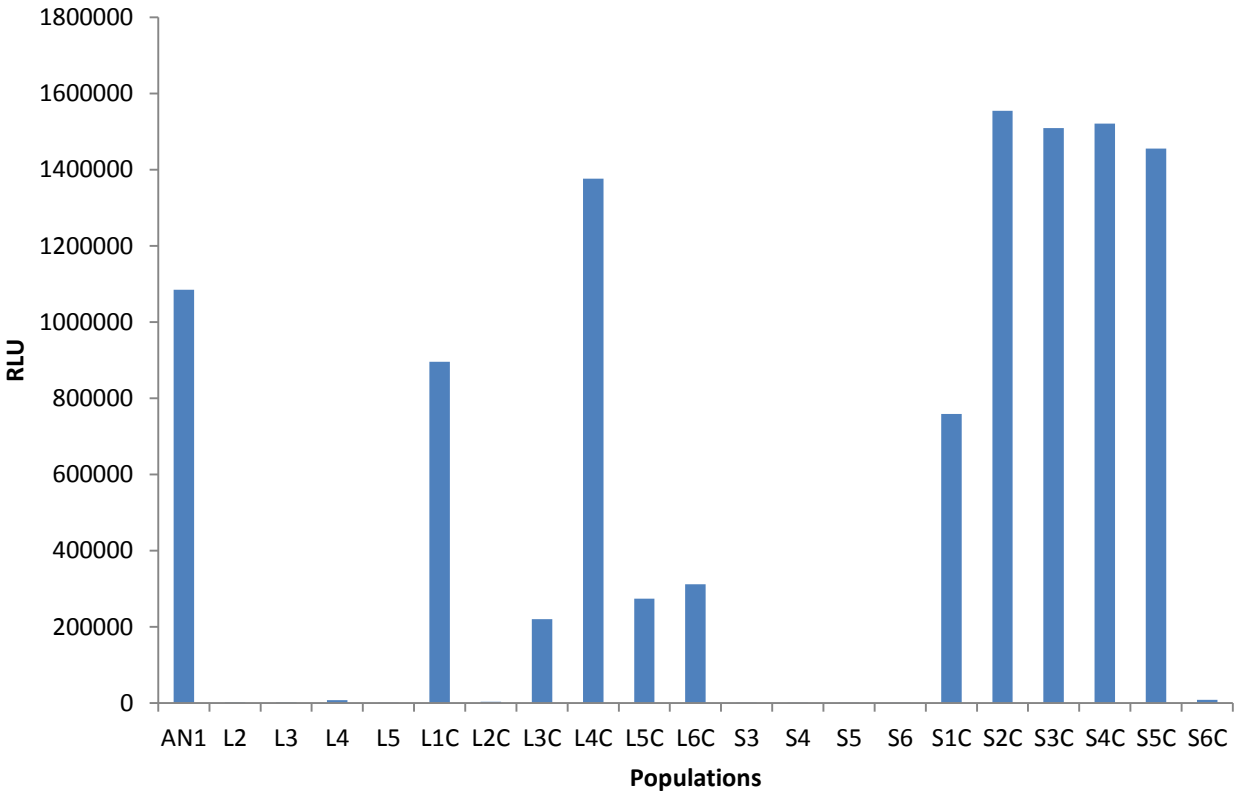


Figure X1.4: Light output from *lasB:lux* promoter fusion, evolved populations. The data presented here is an area under the curve calculation, extrapolated from the raw TECAN data.

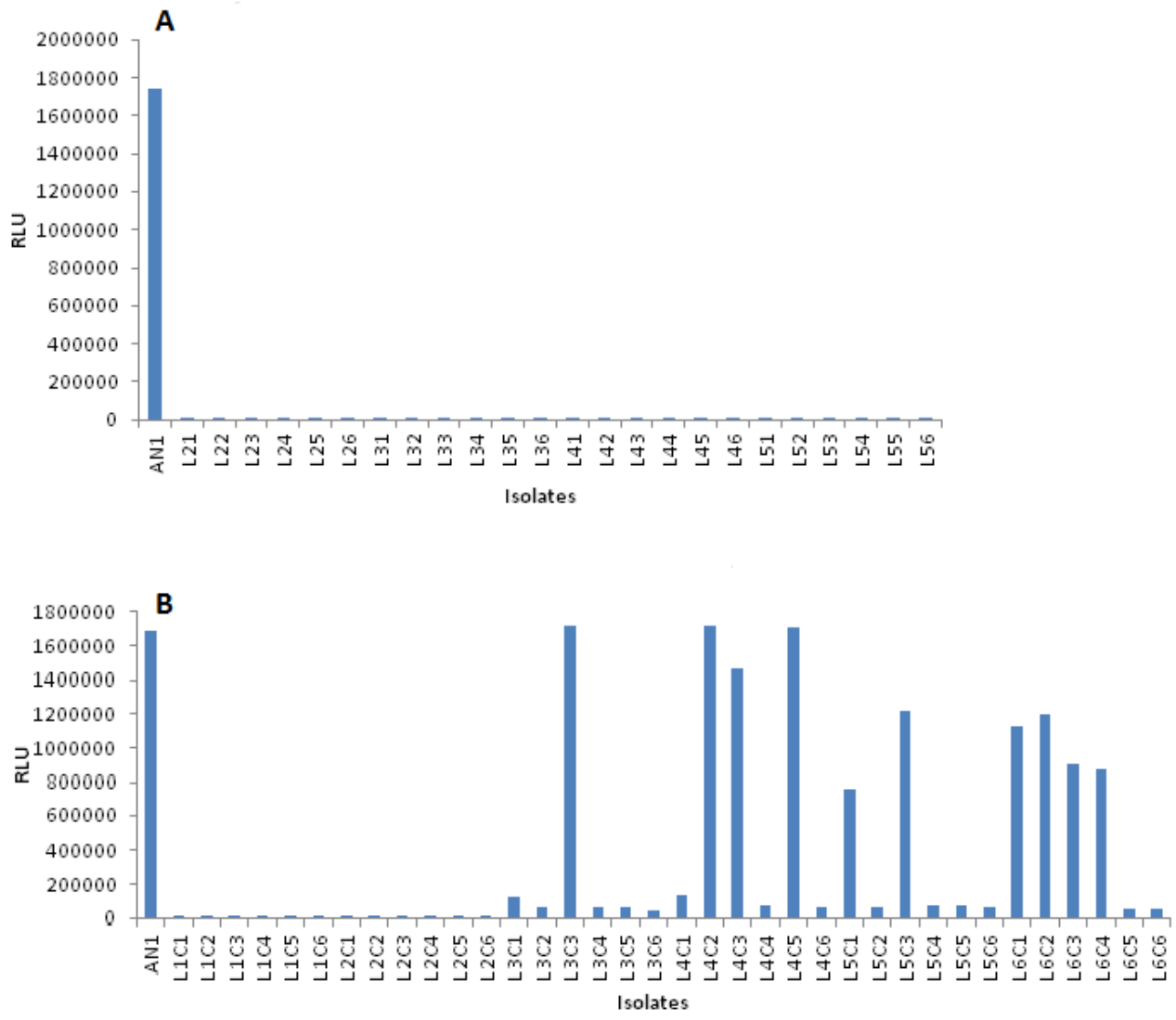


Figure X1.5: Light output from *lasB:lux* promoter fusion, low host density, worm evolved isolates (A) and control isolates (B). The data presented here is an area under the curve calculation, extrapolated from the raw TECAN data.

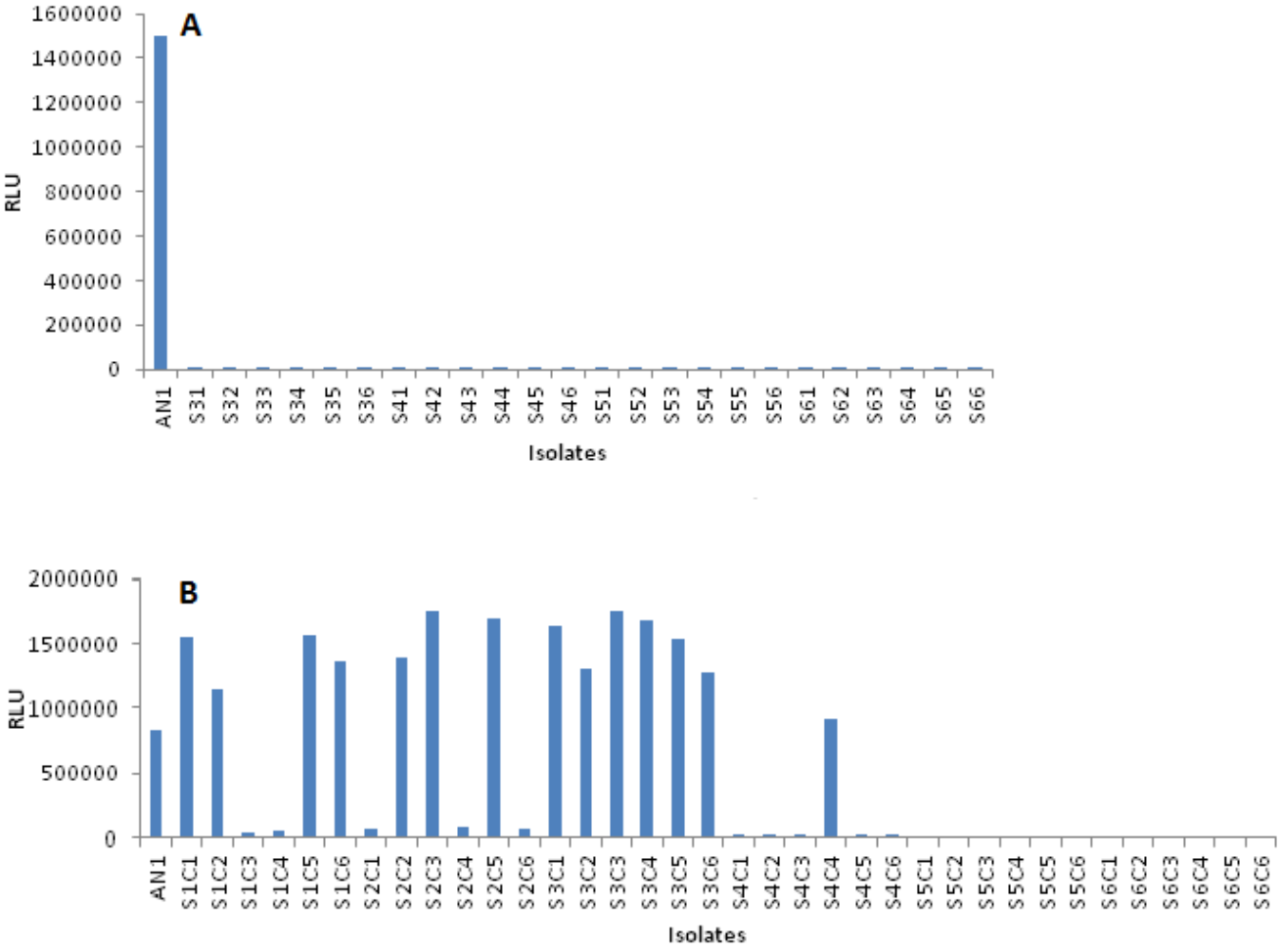


Figure X1.6: Light output from *lasB:lux* promoter fusion, high host density, worm evolved isolates(A) and control isolates (B). The data presented here is an area under the curve calculation, extrapolated from the raw TECAN data.

Appendix 2: *P. aeruginosa* evolved strain list, *G. mellonella* and *V. radiata* data sets

Strain	Description	Source
Populations		
L2	Low Host Density *P. aeruginosa* population 2, evolved from AN1.	This Study
L3	Low Host Density *P. aeruginosa* population 3, evolved from AN1.	This Study
L4	Low Host Density *P. aeruginosa* population 4, evolved from AN1.	This Study
L5	Low Host Density *P. aeruginosa* population 5, evolved from AN1.	This Study
L1C	Low Host Density *P. aeruginosa* control population 1, evolved from AN1.	This Study
L2C	Low Host Density *P. aeruginosa* control population 2, evolved from AN1.	This Study
L3C	Low Host Density *P. aeruginosa* control population 3, evolved from AN1.	This Study
L4C	Low Host Density *P. aeruginosa* control population 4, evolved from AN1.	This Study
L5C	Low Host Density *P. aeruginosa* control population 5, evolved from AN1.	This Study
L6C	Low Host Density *P. aeruginosa* control population 6, evolved from AN1.	This Study
S3	High Host Density P. *aeruginosa* population 3, evolved from AN1.	This Study
S4	High Host Density *P. aeruginosa* population 4, evolved from AN1.	This Study
S5	High Host Density *P. aeruginosa* population 5, evolved from AN1.	This Study
S6	High Host Density *P. aeruginosa* population 6, evolved from AN1.	This Study
S1C	High Host Density *P. aeruginosa* control population 1, evolved from AN1.	This Study
S2C	High Host Density *P. aeruginosa* control population 2, evolved from AN1.	This Study
S3C	High Host Density *P. aeruginosa* control population 3, evolved from AN1.	This Study
S4C	High Host Density *P. aeruginosa* control population	This Study

Strain	Description	Source
	4, evolved from AN1.	
S5C	High Host Density *P. aeruginosa* control population 5, evolved from AN1.	This Study
S6C	High Host Density *P. aeruginosa* control population 6, evolved from AN1.	This Study
Individual Isolates		
L21	First Isolate from L2 population.	This Study
L22	Second Isolate from L2 population.	This Study
L23	Third Isolate from L2 population.	This Study
L24	Fourth Isolate from L2 population.	This Study
L25	Fifth Isolate from L2 population.	This Study
L26	Sixth Isolate from L2 population.	This Study
L31	First Isolate from L3 population.	This Study
L32	Second Isolate from L3 population.	This Study
L33	Third Isolate from L3 population.	This Study
L34	Fourth Isolate from L3 population.	This Study
L35	Fifth Isolate from L3 population.	This Study
L36	Sixth Isolate from L3 population.	This Study
L41	First Isolate from L4 population.	This Study
L42	Second Isolate from L4 population.	This Study
L43	Third Isolate from L4 population.	This Study
L44	Fourth Isolate from L4 population.	This Study
L45	Fifth Isolate from L4 population.	This Study
L46	Sixth Isolate from L4 population.	This Study
L51	First Isolate from L5 population.	This Study
L52	Second Isolate from L5 population.	This Study
L53	Third Isolate from L5 population.	This Study
L54	Fourth Isolate from L5 population.	This Study
L55	Fifth Isolate from L5 population.	This Study
L56	Sixth Isolate from L5 population.	This Study
L1C1	First Isolate from L1C population.	This Study
L1C2	Second Isolate from L1C population.	This Study
L1C3	Third Isolate from L1C population.	This Study
L1C4	Fourth Isolate from L1C population.	This Study
L1C5	Fifth Isolate from L1C population.	This Study
L1C6	Sixth Isolate from L1C population.	This Study
L2C1	First Isolate from L2C population.	This Study

Strain	Description	Source
L2C2	Second Isolate from L2C population.	This Study
L2C3	Third Isolate from L2C population.	This Study
L2C4	Fourth Isolate from L2C population.	This Study
L2C5	Fifth Isolate from L2C population.	This Study
L2C6	Sixth Isolate from L2C population.	This Study
L3C1	First Isolate from L3C population.	This Study
L3C2	Second Isolate from L3C population.	This Study
L3C3	Third Isolate from L3C population.	This Study
L3C4	Fourth Isolate from L3C population.	This Study
L3C5	Fifth Isolate from L3C population.	This Study
L3C6	Sixth Isolate from L3C population.	This Study
L4C1	First Isolate from L4C population.	This Study
L4C2	Second Isolate from L4C population.	This Study
L4C3	Third Isolate from L4C population.	This Study
L4C4	Fourth Isolate from L4C population.	This Study
L4C5	Fifth Isolate from L4C population.	This Study
L4C6	Sixth Isolate from L4C population.	This Study
L5C1	First Isolate from L5C population.	This Study
L5C2	Second Isolate from L5C population.	This Study
L5C3	Third Isolate from L5C population.	This Study
L5C4	Fourth Isolate from L5C population.	This Study
L5C5	Fifth Isolate from L5C population.	This Study
L5C6	Sixth Isolate from L5C population.	This Study
L6C1	First Isolate from L6C population.	This Study
L6C2	Second Isolate from L6C population.	This Study
L6C3	Third Isolate from L6C population.	This Study
L6C4	Fourth Isolate from L6C population.	This Study
L6C5	Fifth Isolate from L6C population.	This Study
L6C6	Sixth Isolate from L6C population.	This Study
S31	First Isolate from S3 population.	This Study
S32	Second Isolate from S3 population.	This Study
S33	Third Isolate from S3 population.	This Study
S34	Fourth Isolate from S3 population.	This Study
S35	Fifth Isolate from S3 population.	This Study
S36	Sixth Isolate from S3 population.	This Study
S41	First Isolate from S4 population.	This Study

Strain	Description	Source
S42	Second Isolate from S4 population.	This Study
S43	Third Isolate from S4 population.	This Study
S44	Fourth Isolate from S4 population.	This Study
S45	Fifth Isolate from S4 population.	This Study
S46	Sixth Isolate from S4 population.	This Study
S51	First Isolate from S5 population.	This Study
S52	Second Isolate from S5 population.	This Study
S53	Third Isolate from S5 population.	This Study
S54	Fourth Isolate from S5 population.	This Study
S55	Fifth Isolate from S5 population.	This Study
S56	Sixth Isolate from S5 population.	This Study
S61	First Isolate from S6 population.	This Study
S62	Second Isolate from S6 population.	This Study
S63	Third Isolate from S6 population.	This Study
S64	Fourth Isolate from S6 population.	This Study
S65	Fifth Isolate from S6 population.	This Study
S66	Sixth Isolate from S6 population.	This Study
S1C1	First Isolate from S1C population.	This Study
S1C2	Second Isolate from S1C population.	This Study
S1C3	Third Isolate from S1C population.	This Study
S1C4	Fourth Isolate from S1C population.	This Study
S1C5	Fifth Isolate from S1C population.	This Study
S1C6	Sixth Isolate from S1C population.	This Study
S2C1	First Isolate from S2C population.	This Study
S2C2	Second Isolate from S2C population.	This Study
S2C3	Third Isolate from S2C population.	This Study
S2C4	Fourth Isolate from S2C population.	This Study
S2C5	Fifth Isolate from S2C population.	This Study
S2C6	Sixth Isolate from S2C population.	This Study
S3C1	First Isolate from S3C population.	This Study
S3C2	Second Isolate from S3C population.	This Study
S3C3	Third Isolate from S3C population.	This Study
S3C4	Fourth Isolate from S3C population.	This Study
S3C5	Fifth Isolate from S3C population.	This Study
S3C6	Sixth Isolate from S3C population.	This Study
S4C1	First Isolate from S4C population.	This Study

Strain	Description	Source
S4C2	Second Isolate from S4C population.	This Study
S4C3	Third Isolate from S4C population.	This Study
S4C4	Fourth Isolate from S4C population.	This Study
S4C5	Fifth Isolate from S4C population.	This Study
S4C6	Sixth Isolate from S4C population.	This Study
S5C1	First Isolate from S5C population.	This Study
S5C2	Second Isolate from S5C population.	This Study
S5C3	Third Isolate from S5C population.	This Study
S5C4	Forth Isolate from S5C population.	This Study
S5C5	Fifth Isolate from S5C population.	This Study
S5C6	Sixth Isolate from S5C population.	This Study
S6C1	First Isolate from S6C population.	This Study
S6C2	Second Isolate from S5C population.	This Study
S6C3	Third Isolate from S5C population.	This Study
S6C4	Fourth Isolate from S5C population.	This Study
S6C5	Fifth Isolate from S5C population.	This Study
S6C6	Sixth Isolate from S5C population.	This Study

Table X2.1: Summary of evolved *P. aeruginosa* populations and isolates produced during this study.

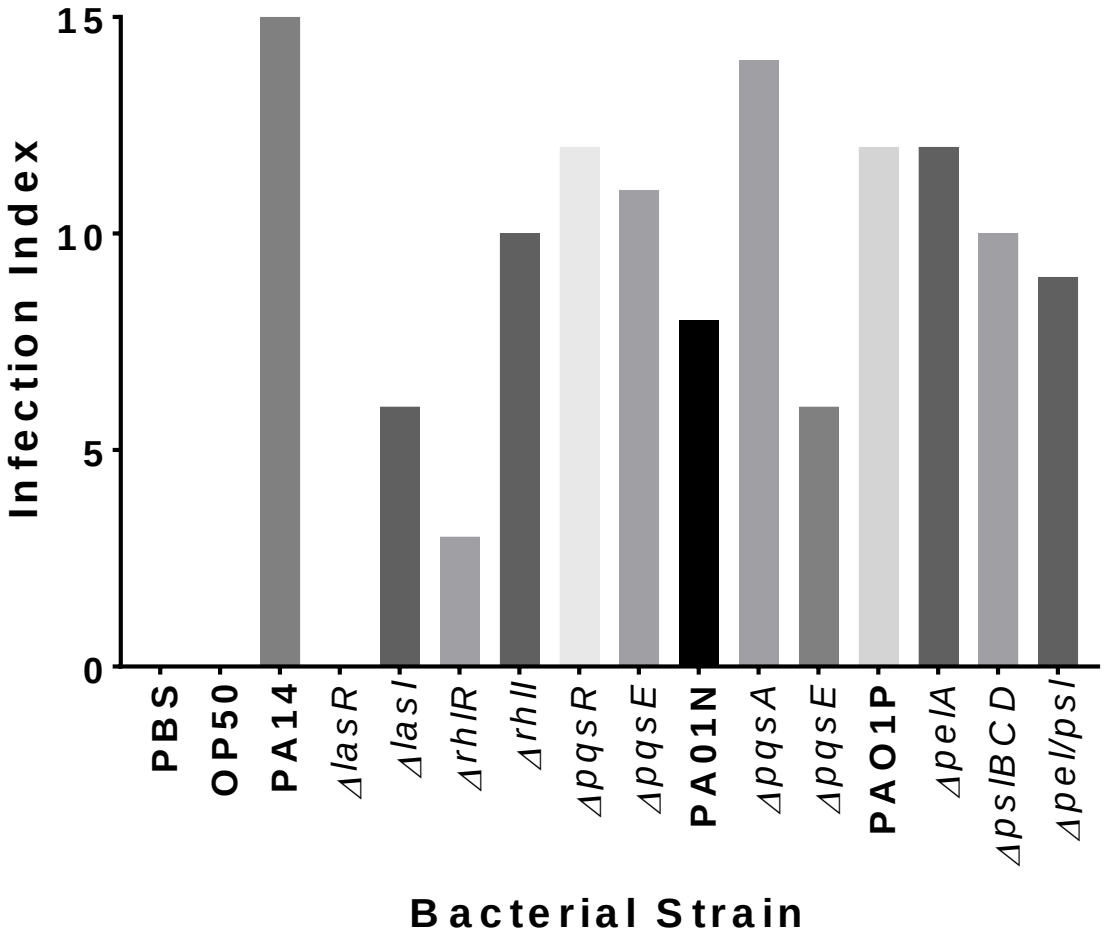


Figure X2.1: Infection index from *P. aeruginosa* laboratory strain and mutant *G. mellonella* assay.

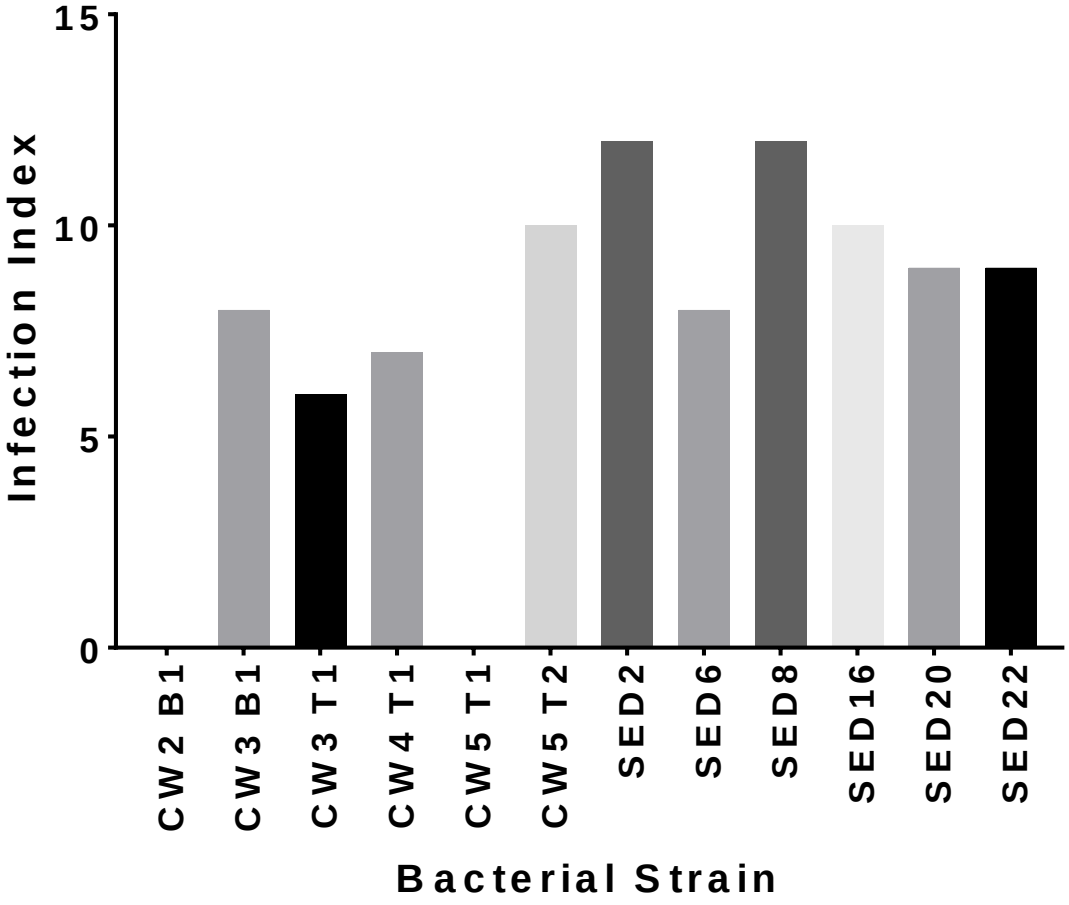


Figure X2: Infection index from CF and CW clinical isolate *G. mellonella* assay.

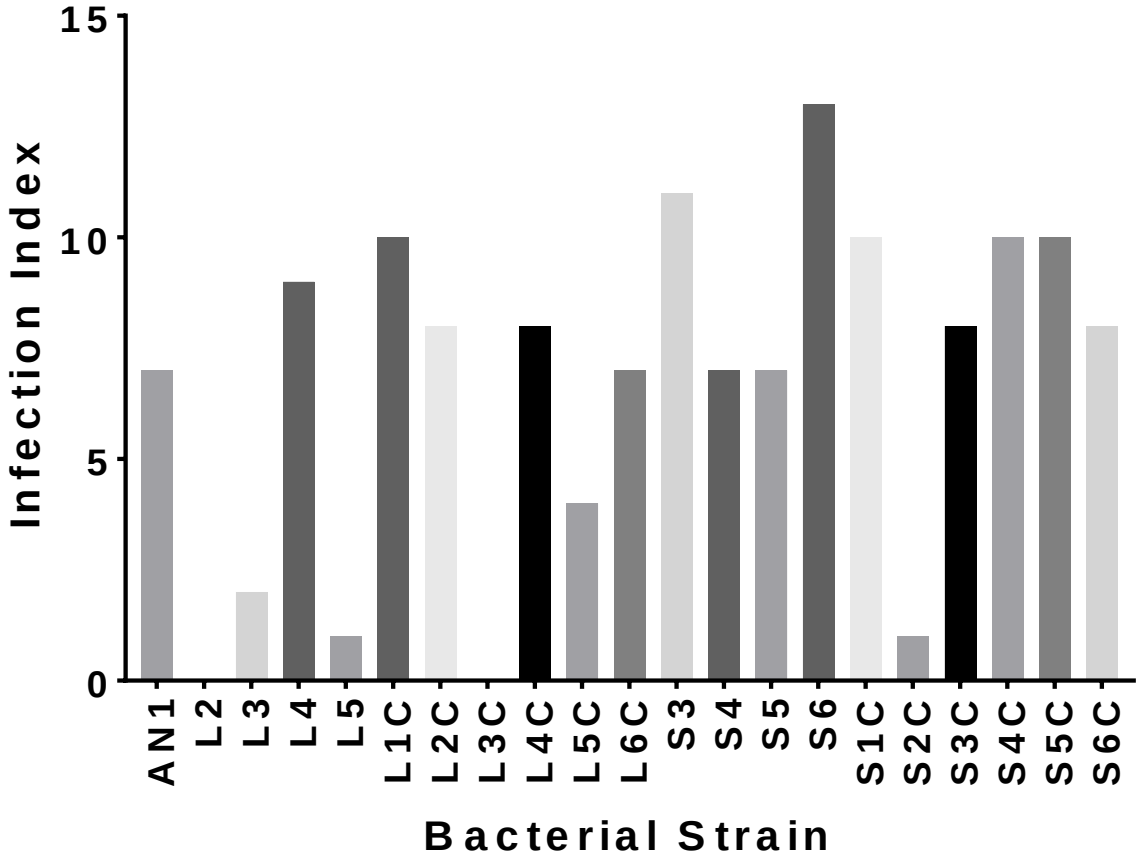


Figure X2.3: Infection index from evolved populations *G. mellonella* assay.

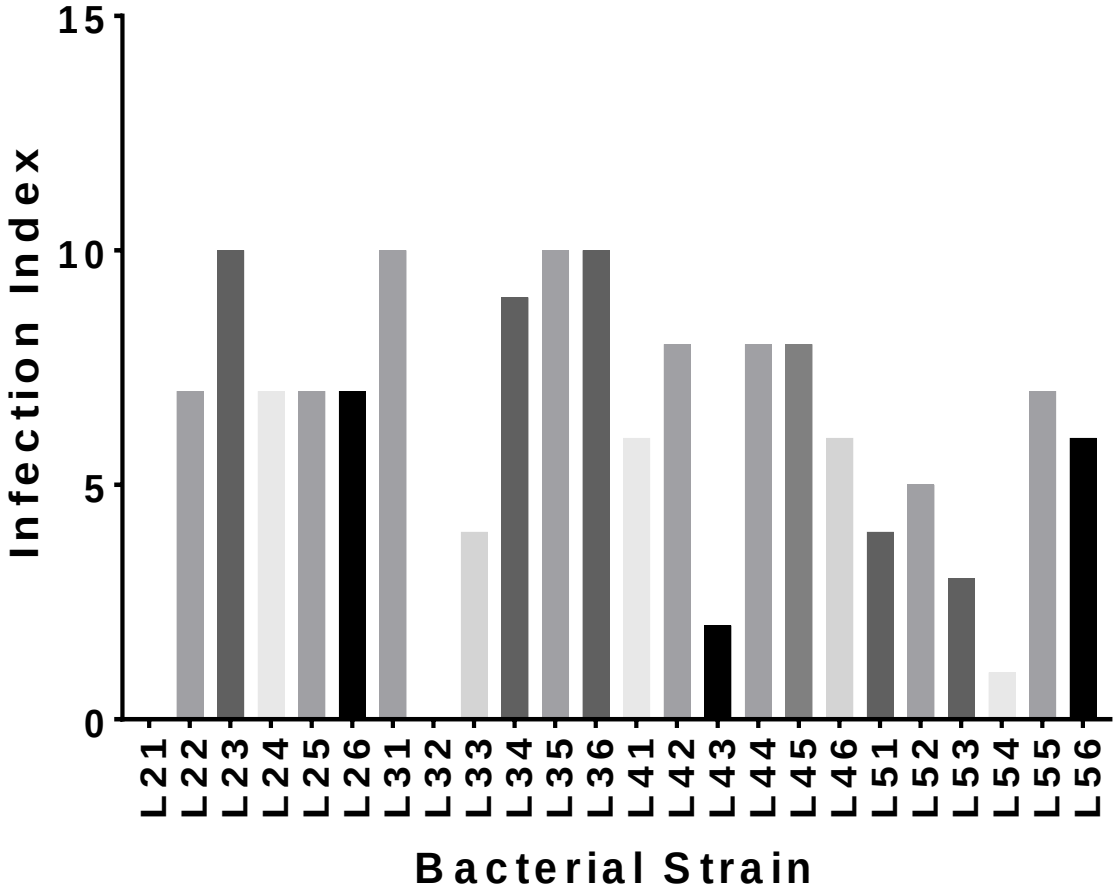


Figure X2.4: Infection index from low host density, worm evolved isolates *G. mellonella* assay.

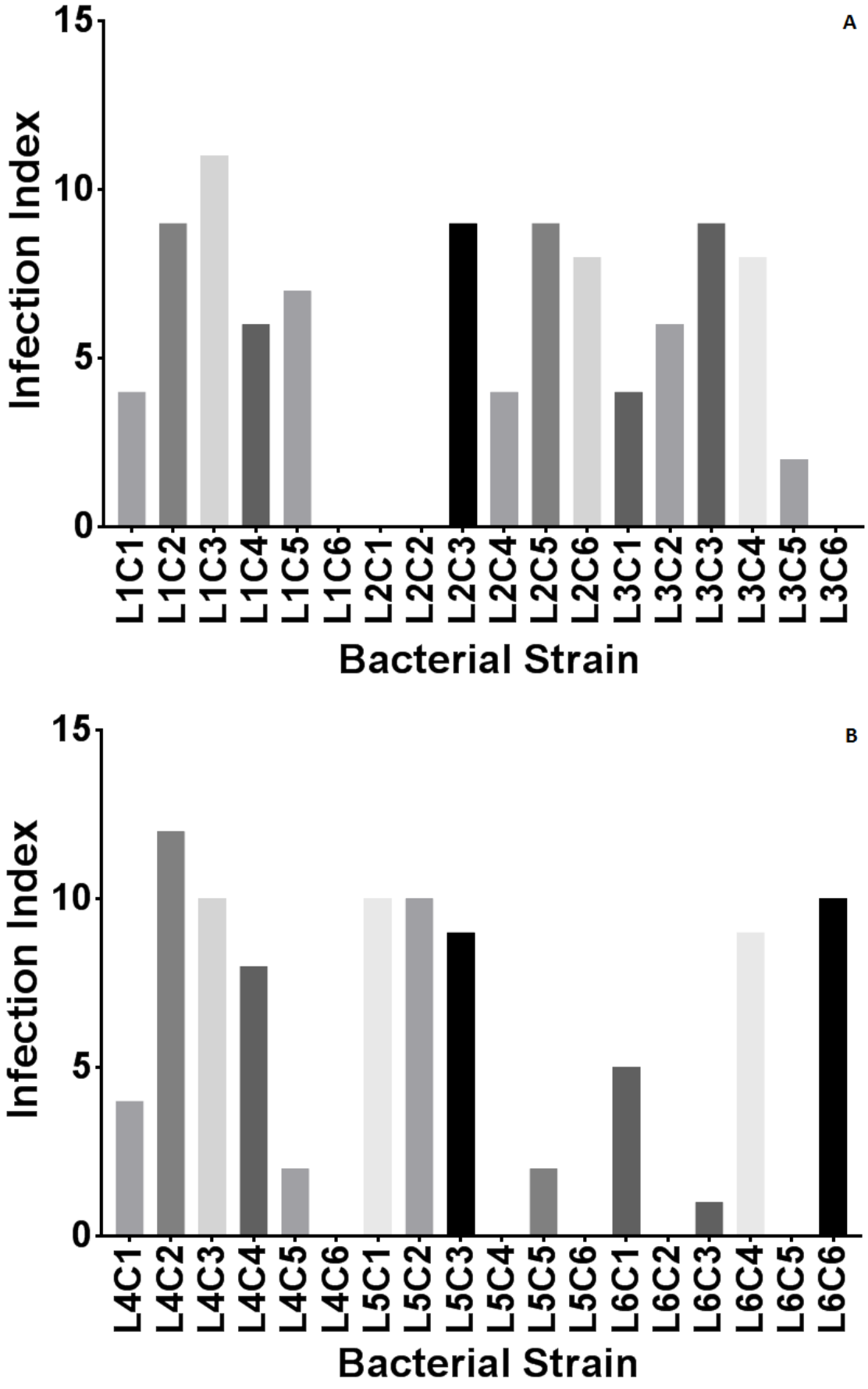


Figure X2.5: Infection index from low host density control isolates *G. mellonella* assay.

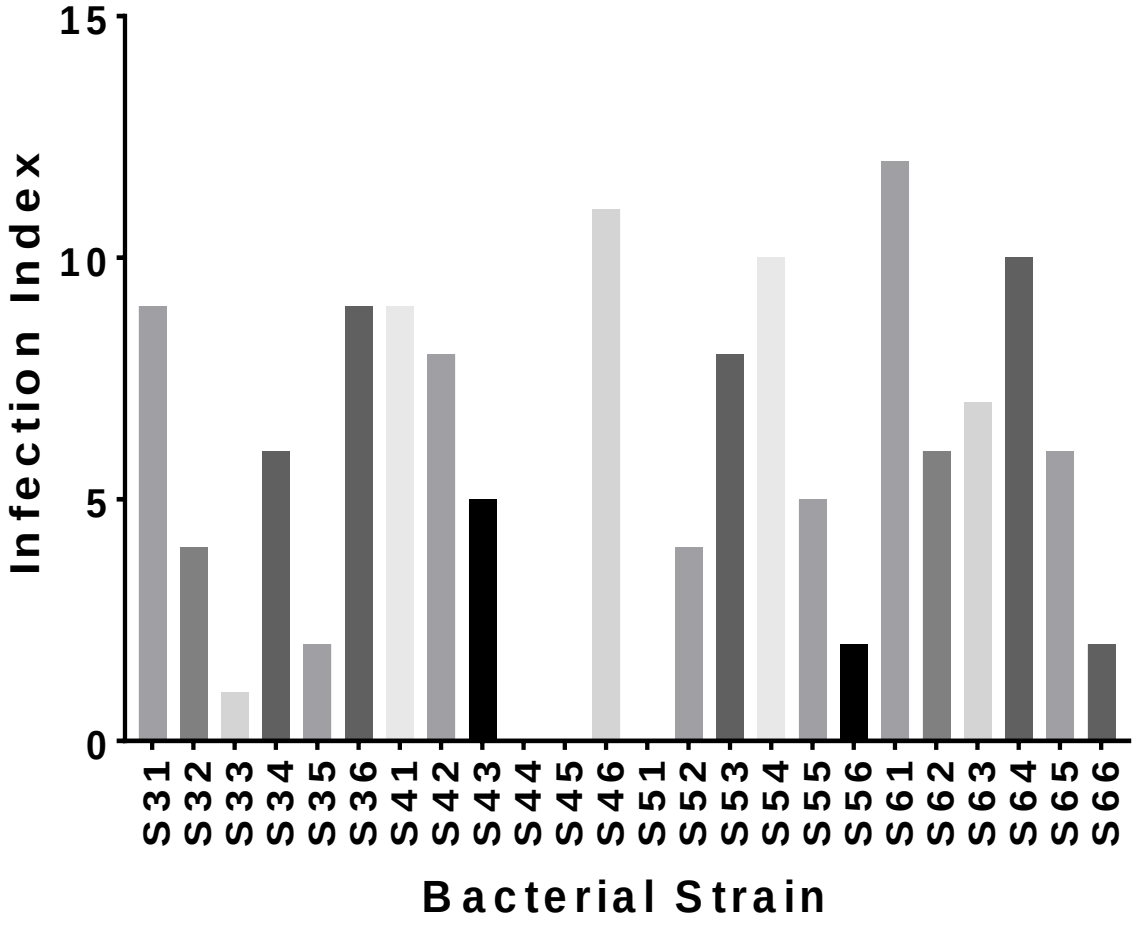


Figure X2.6: Infection index from high host density, worm evolved isolates *G. mellonella* assay.

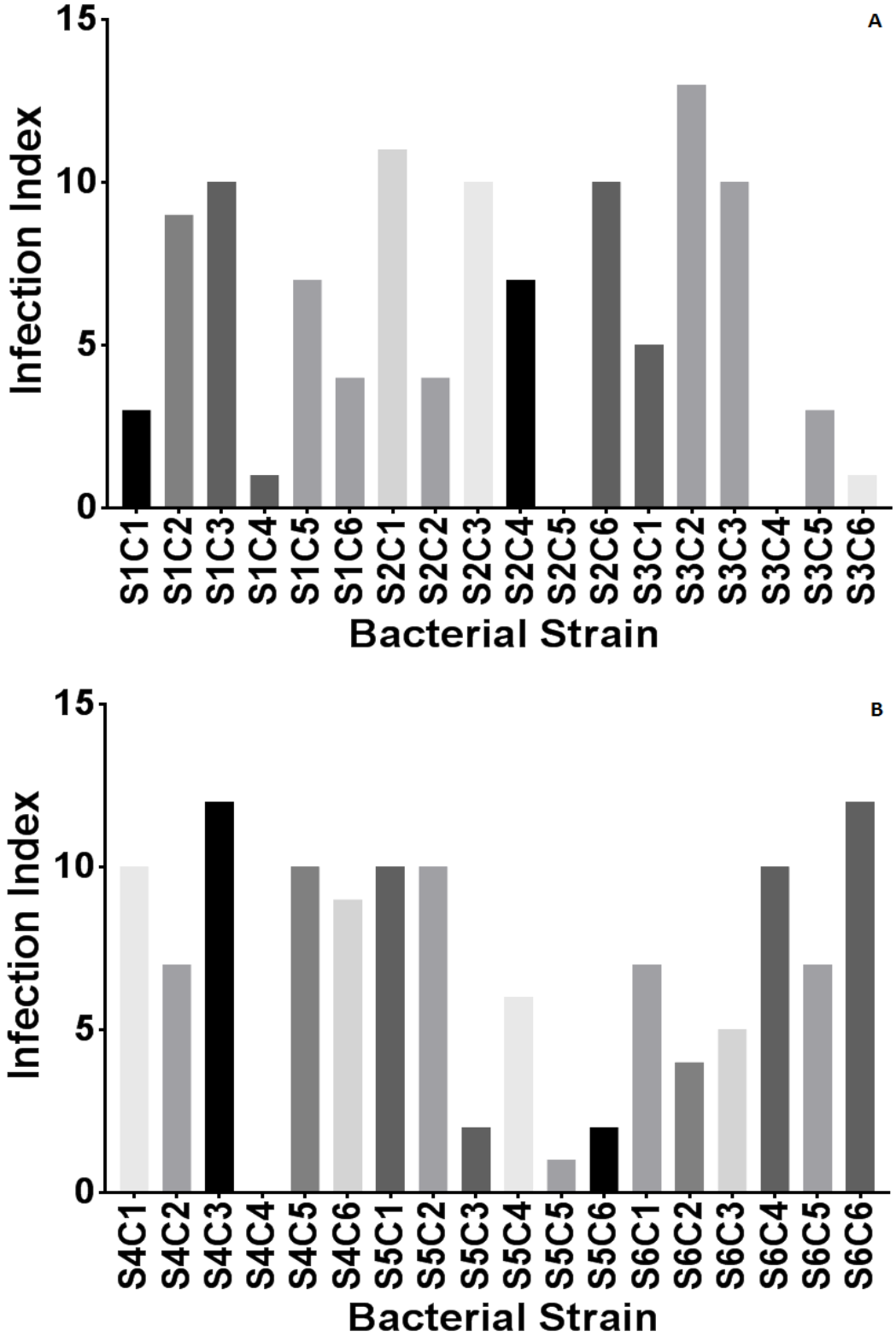


Figure X2.7: Infection index from high host density control isolates *G. mellonella* assay.

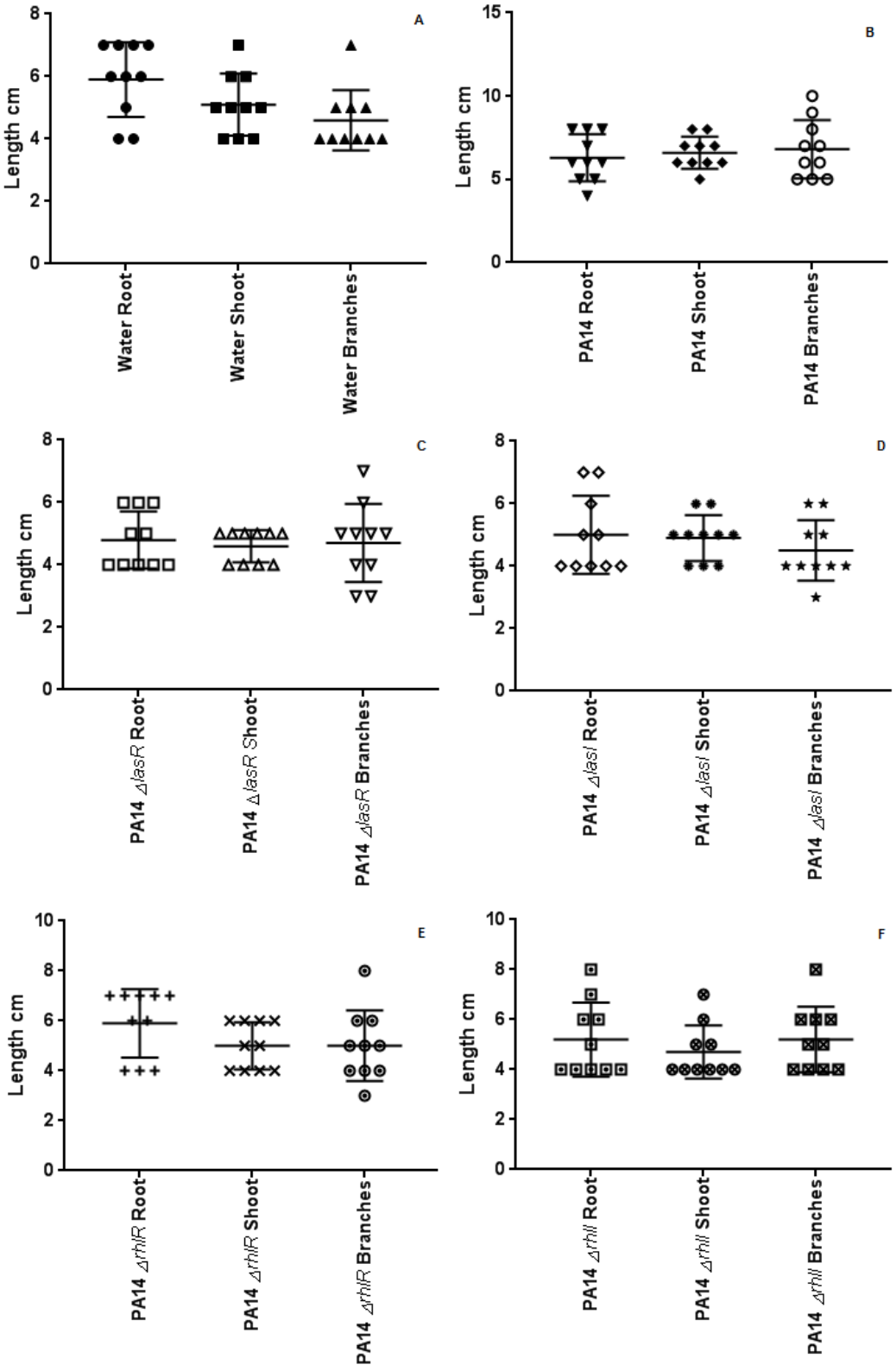


Figure X2.8: Mung bean growth for negative control (A) PA14 wild type (B) and QS mutants (C-F). No *P. aeruginosa* strains attenuated bean growth versus the negative control.

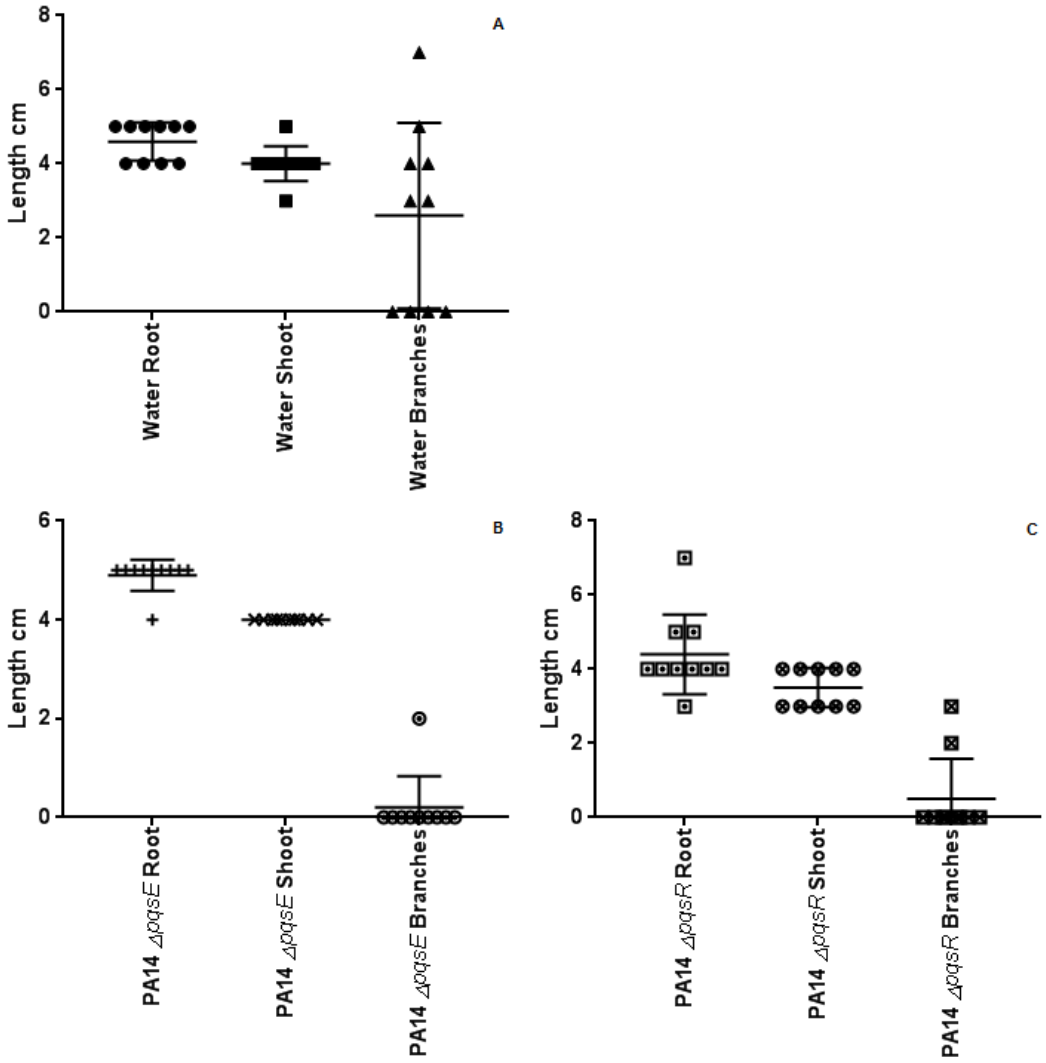


Figure X2.9: Mung bean growth for neagtive control (A) and PA14 *Δpqs* mutants, (B and C). Neither mutant attenuated bean growth compared with the negative control.

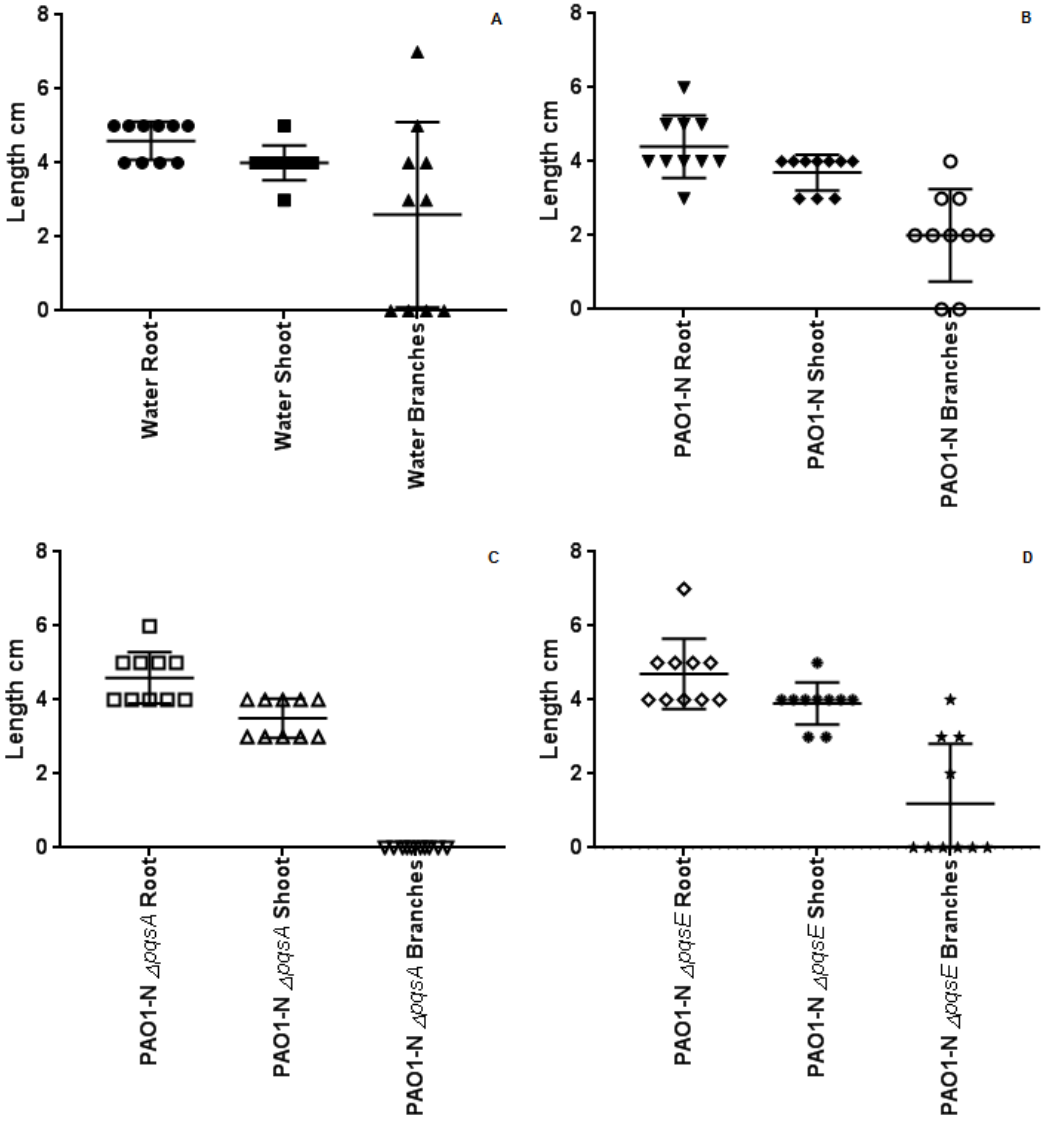


Figure X2.10: Mung bean growth for negative control (A), PAO1-N wild type (B) and *Δpqs* mutants (C and D). No attenuation of bean growth with exposure to *P. aeruginosa* was observed.

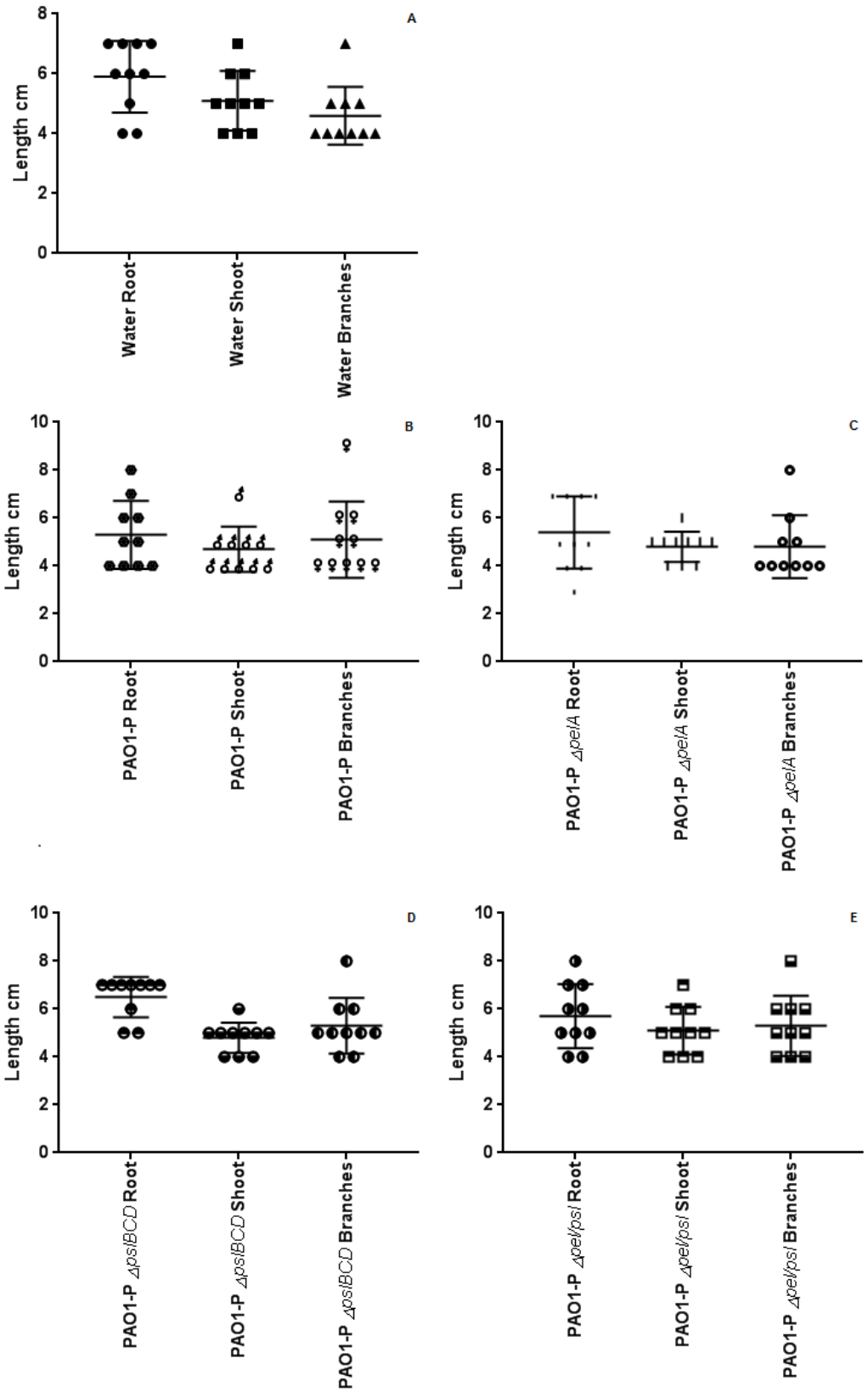


Figure X2.11: Mung bean growth for negative controls (A), PAO1-P wild type (B) and *Δpqs* mutants (C-E). No attenuation of bean growth with exposure to *P. aeruginosa* was observed.

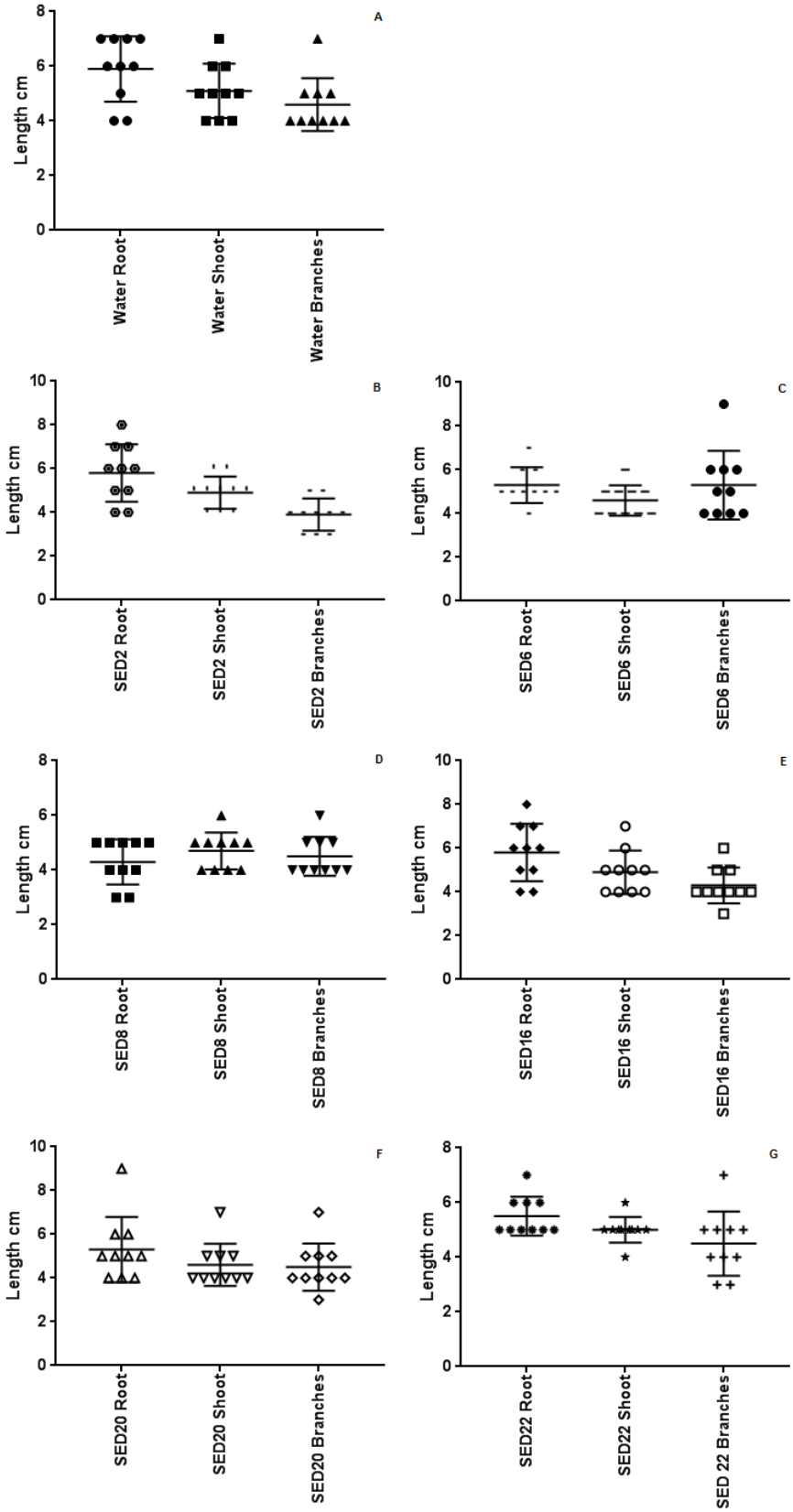


Figure X2.12: Mung bean growth for neagtive control (A) and cystic fibrosis clinical isolates (B-G). All isolates displayed bean growth comparable to the negative control.

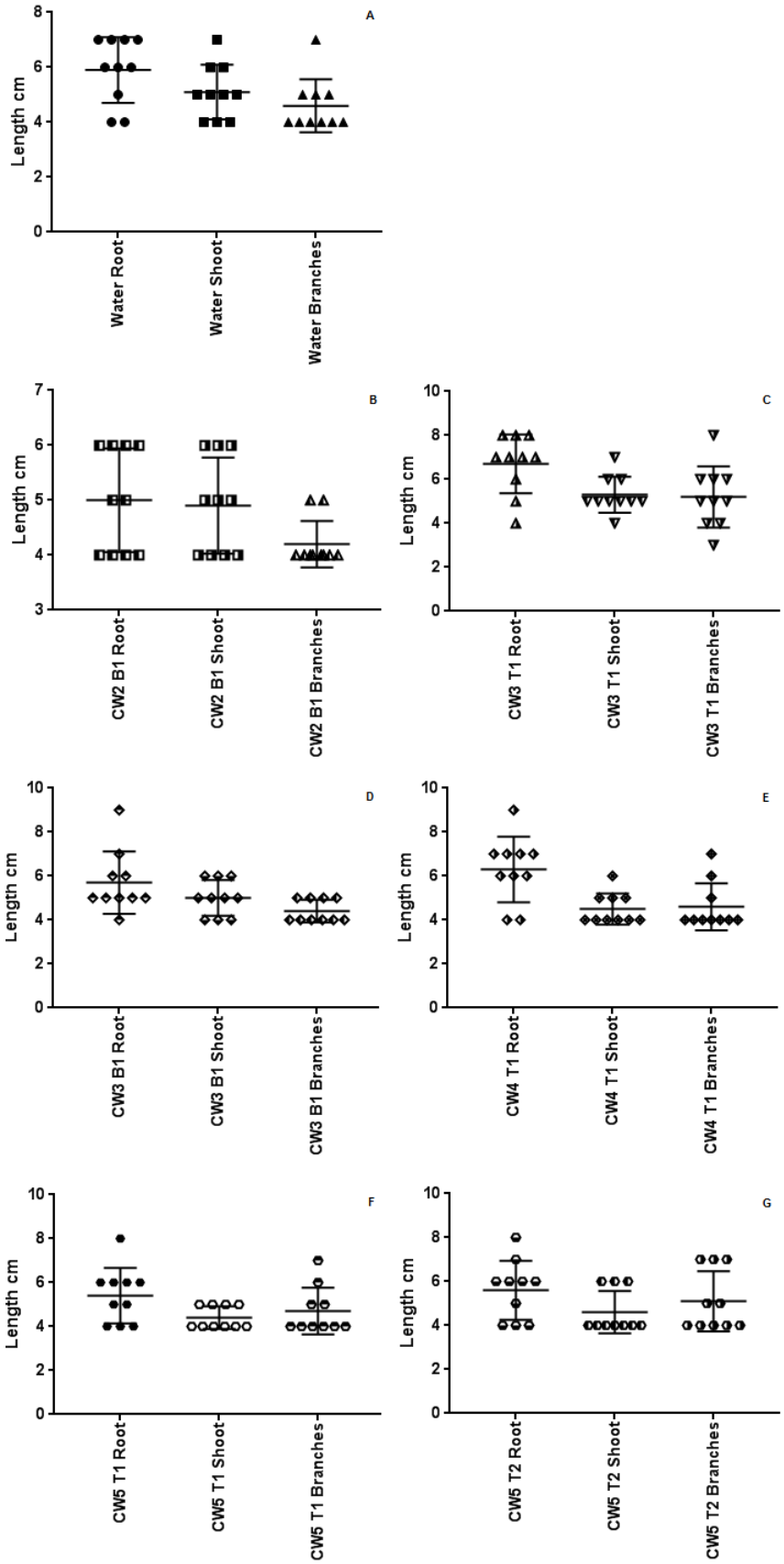


Figure X2.13: Mung bean growth for negative control (A) and chronic wound isolates (B-G). All the isolates had levels of bean growth consistent with the negative control.

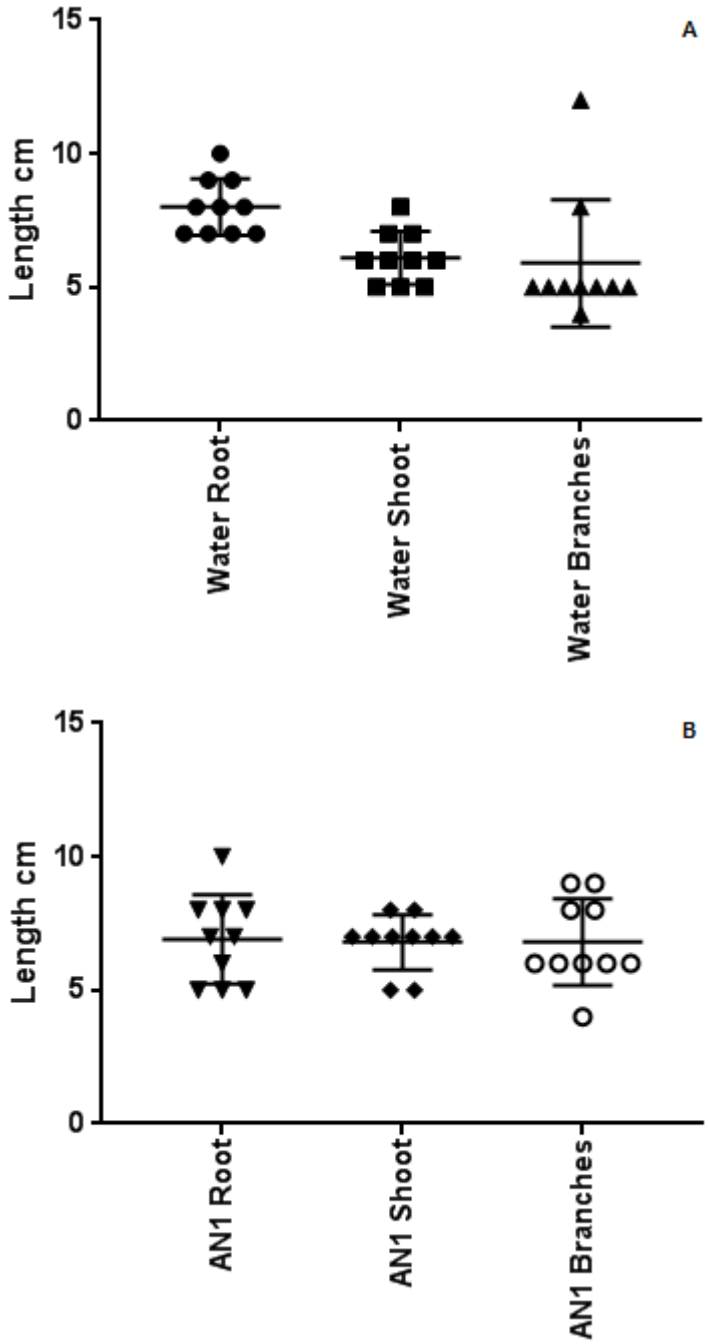


Figure X2.14: Mung bean growth for negative control (A) and AN1 (B). Bean growth was not attenuated when exposed to *P. aeruginosa* compared with the negative control.

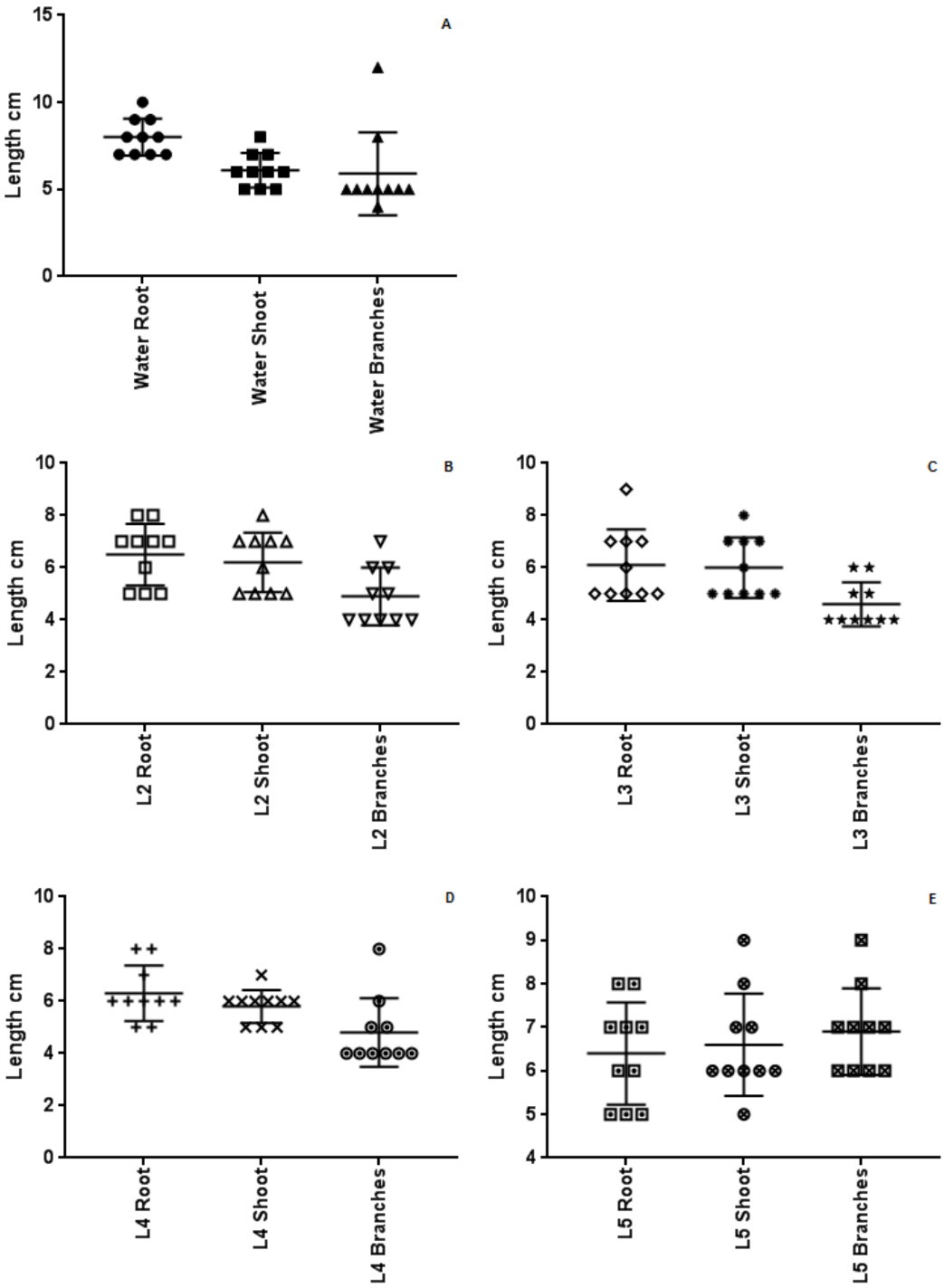


Figure X2.15: Mung bean growth for negative control (A) and low host density worm evolved populations (B-E). Bean growth was not attenuated when exposed to *P. aeruginosa* compared with the negative control.

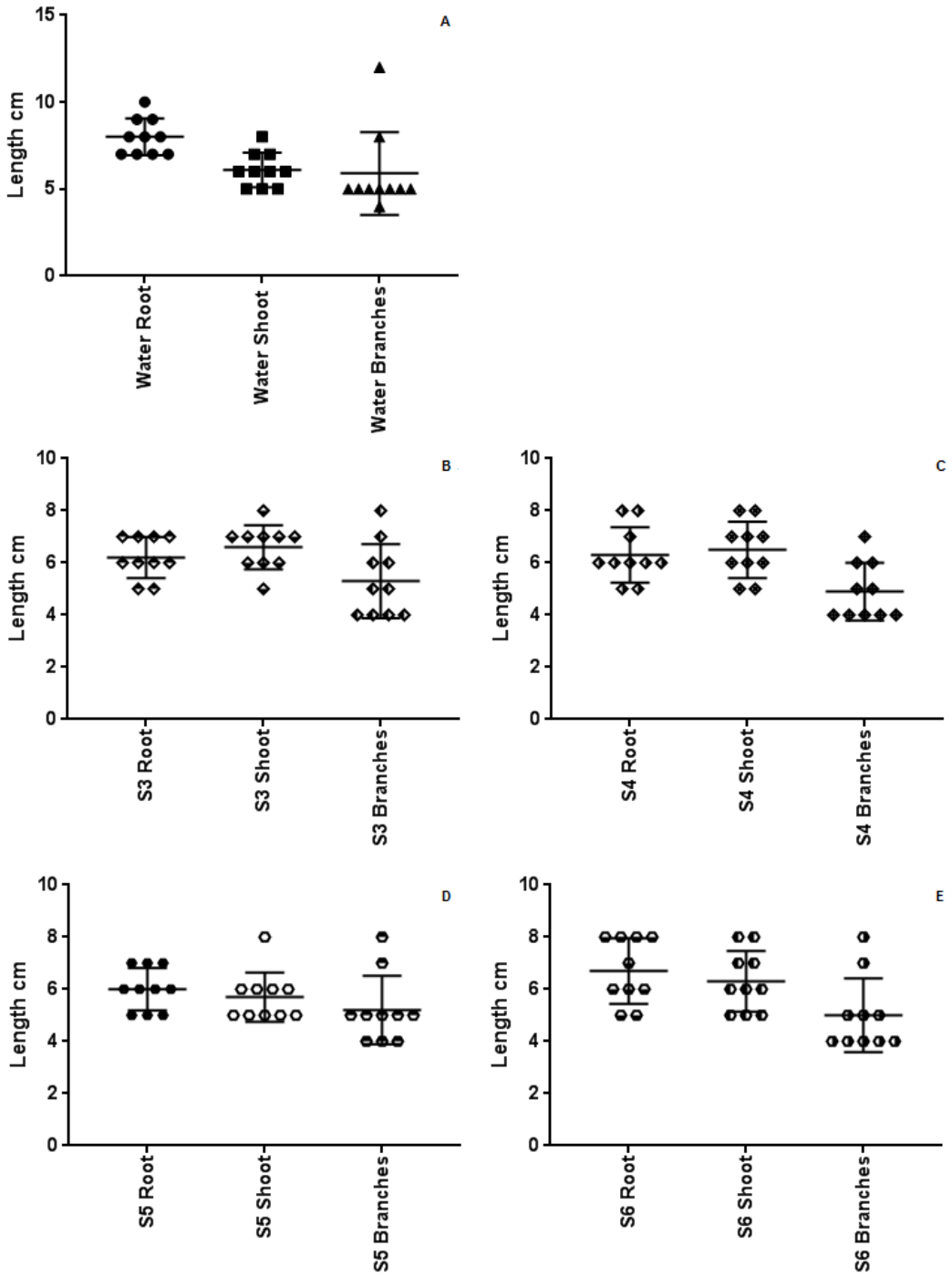


Figure X2.16: Mung bean growth for negative control (A) and high host density worm evolved populations (B-E). All populations displayed bean growth consistent with the negative control.

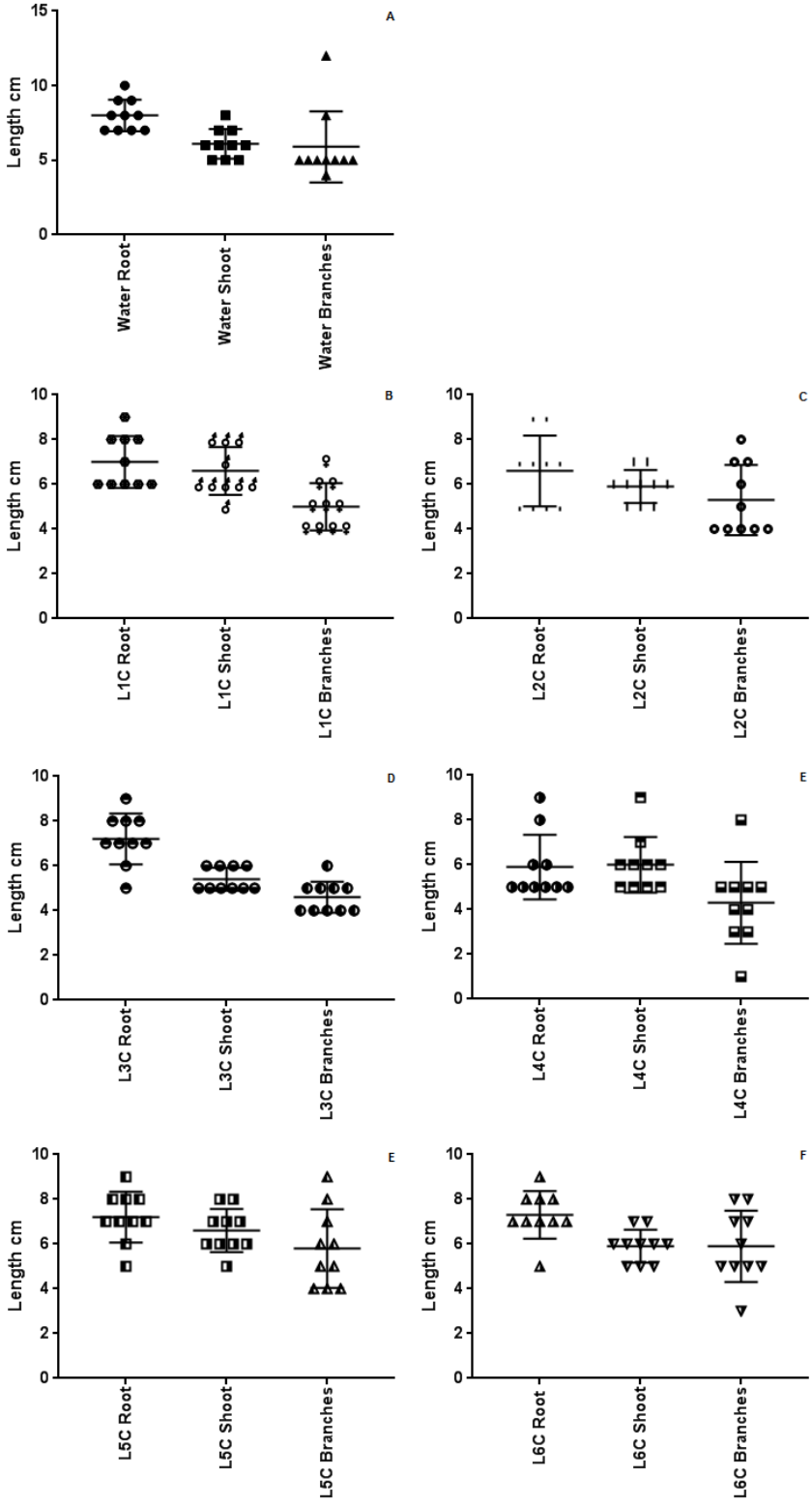


Figure X2.17: Mung bean growth for negative control (A) and low host density control populations (B-F). No attenuation of bean growth was observed for any population.

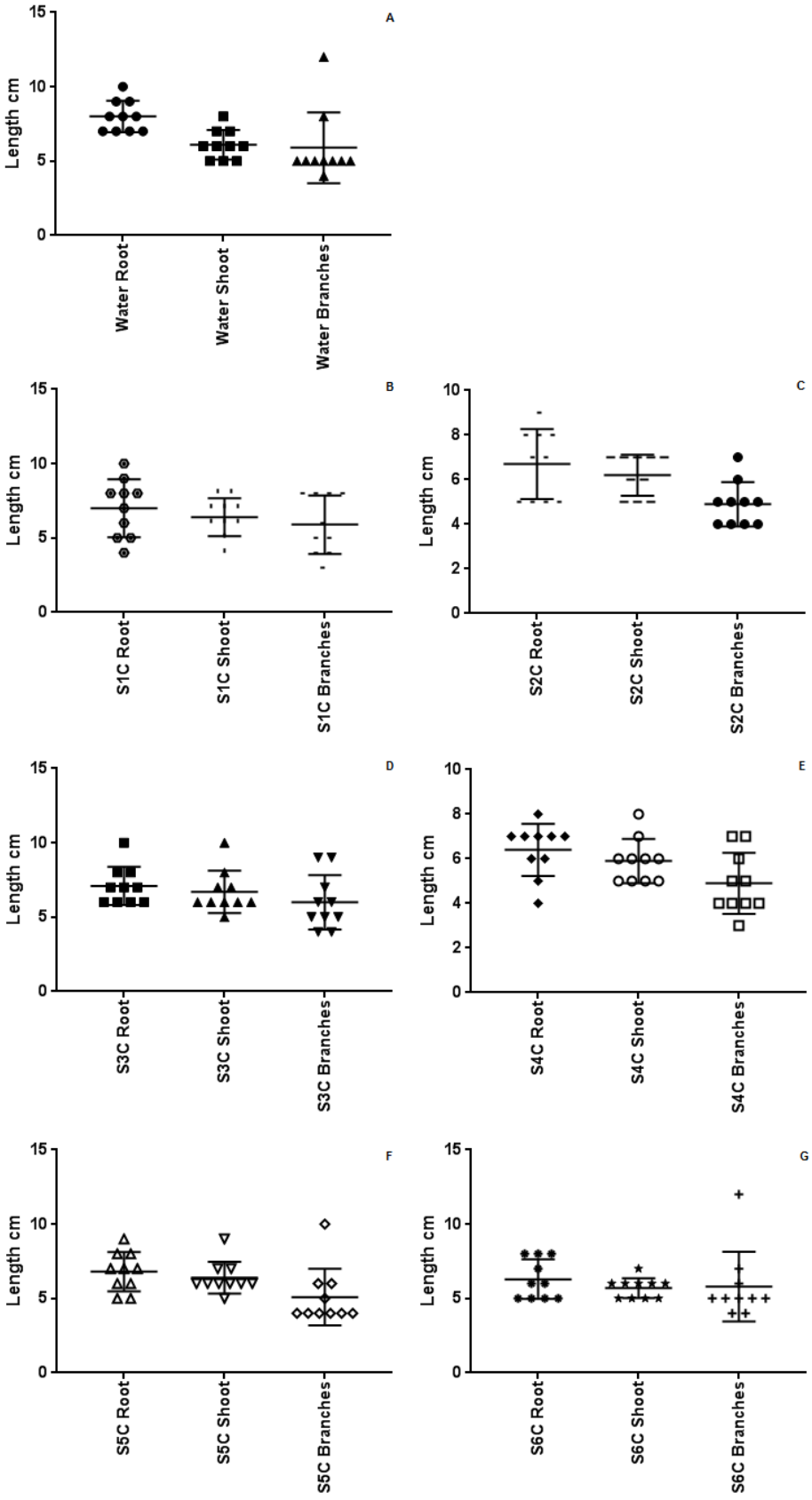


Figure X2:18: Mung bean growth for negative control (A) and high host density control populations (B-G). No attenuation of bean growth was observed for any population.

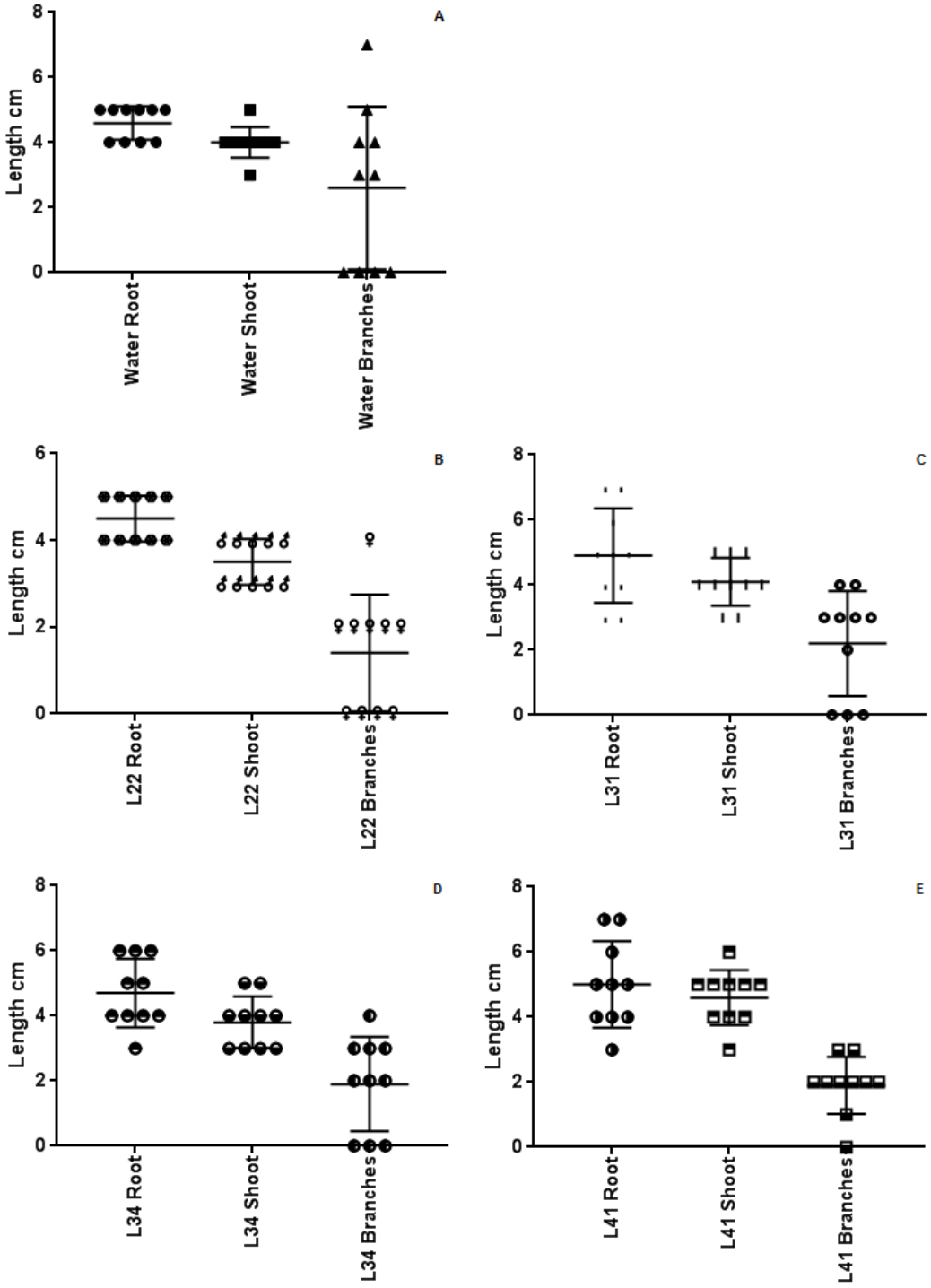


Figure X2.19: Mung bean growth for negative control (A) and chosen low host density worm evolved isolates (B-E). All tested isolates did not display any attenuation of bean growth.

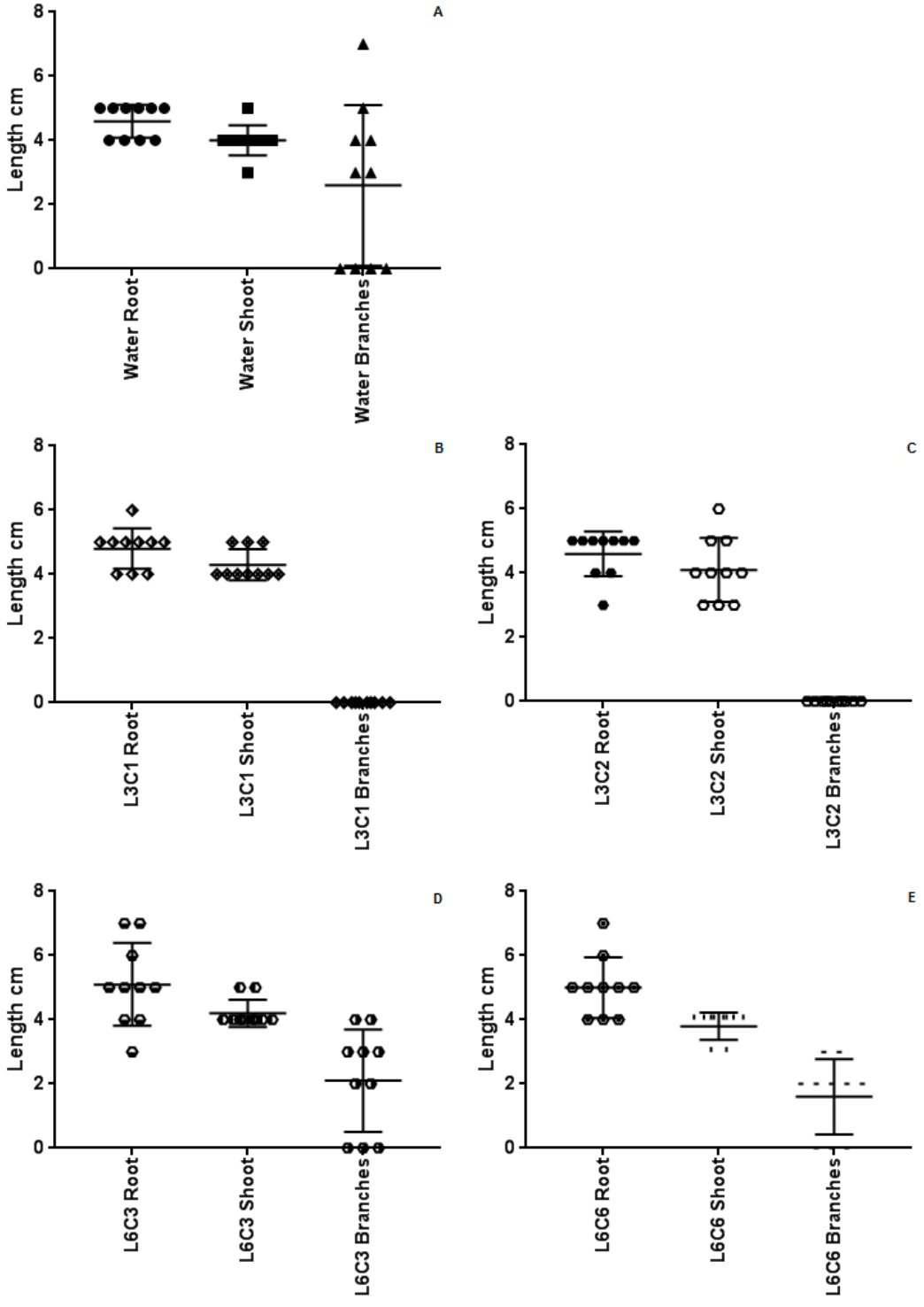


Figure X2.20: Mung bean growth for negative control (A) and chosen low host density control isolates (B-E). No tested isolates attenuated bean growth.

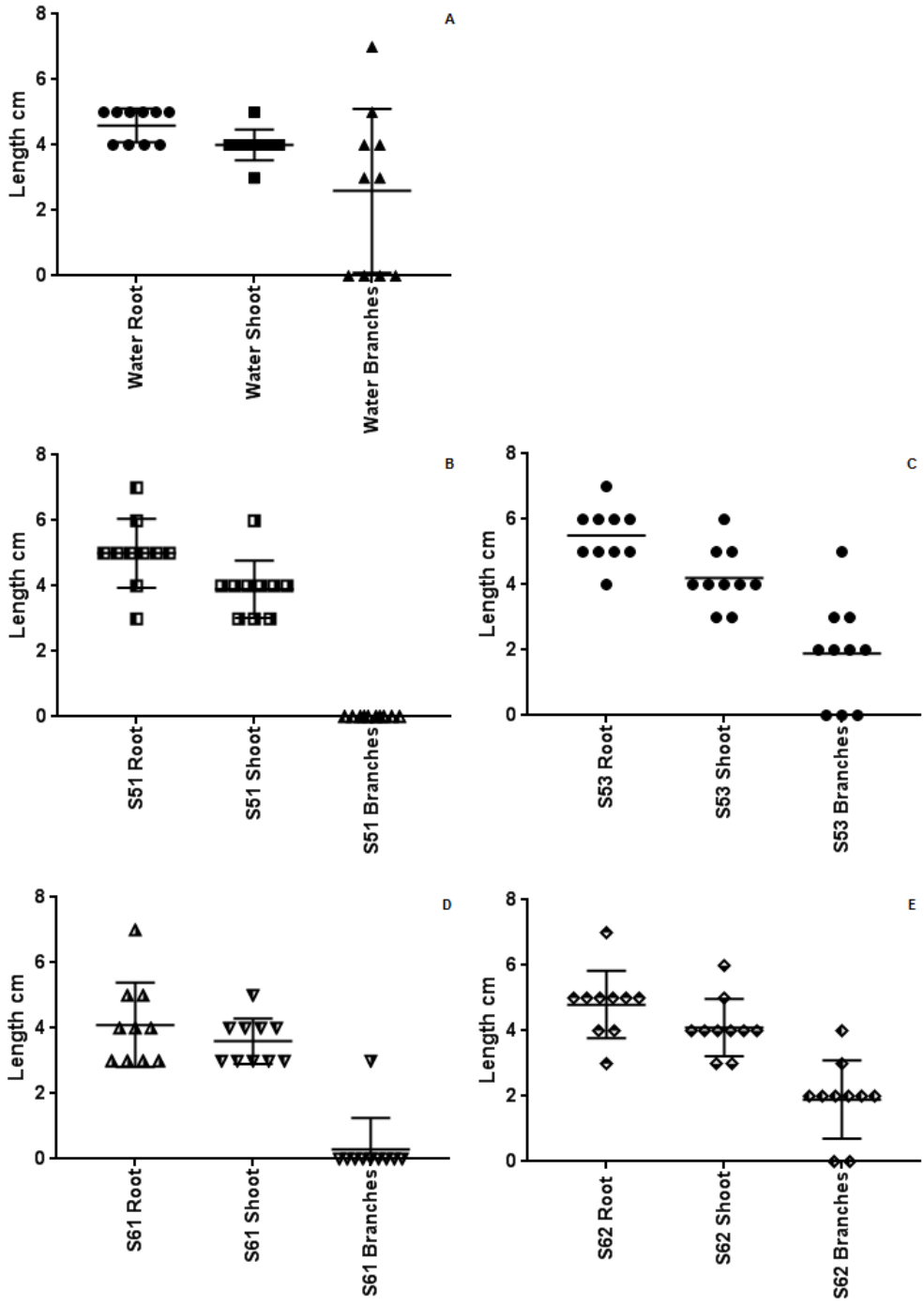


Figure X2.21: Mung bean growth for negative control (A) and chosen high host density worm evolved isolates (B-E). Bean growth was not attenuated in any of the isolates tested. S53 root length increased versus the negative control.

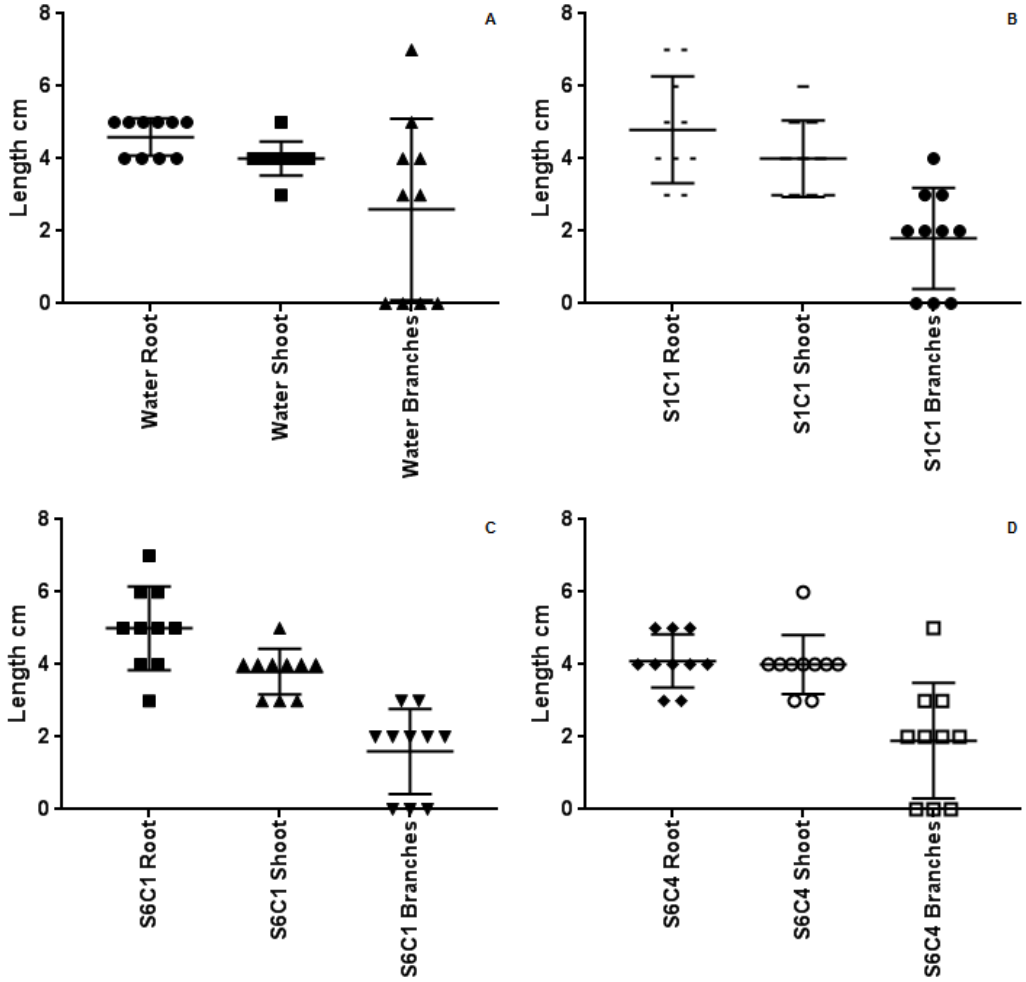


Figure X2.22: Mung bean growth for negative control (A) and chosen high host density control isolates (B-D). All tested isolates were consistent with the negative control, demonstrating no attenuation of bean growth.