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Understanding the impact of phylloplane biocontrol agents on insects

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Abstract

Aphids are a major pest to the agricultural and horticultural industry, causing significant yield losses by directly feeding on crop plants and acting as vectors for plant diseases. Insecticide resistance and concern for the environment and human health has placed growers under increasing pressure to find alternative methods of aphid control. *Pseudomonas poae* PpR24 is a novel potential biocontrol agent with plant-growth promoting properties, proven to be naturally virulent to aphids as well as act as a deterrent when sprayed on a plant. This project furthers our understanding of the impact of PpR24 on the green peach potato aphid, *Myzus persicae*, in a sweet pepper, *Capsicum annumm* L., model system. An experimental evolution approach was applied in a bid to evolve PpR24 isolates with improved aphid virulence and biofilm formation, traits that may be desirable in a biocontrol agent. After ten passage cycles, no improvement was seen in aphicidal properties but one isolate, PpR24b4, evolved significantly strong biofilms at a cost to aphid virulence, bacterial growth and motility. Whole genome sequencing and variant calling analysis of the wild-type and derived isolates found single nucleotide polymorphisms in the genes *wspF* and *gacS* of PpR24b4. Both genes have known associations with biofilm formation and secondary metabolite production, which may explain the phenotypic differences observed between the wild-type and biofilm-forming isolate. To elucidate PpR24s deterrent effect and investigate any changes to volatile emissions as a result of the evolutionary passages, Solid-Phase Microextraction and Dynamic Headspace Extraction with GC-MS were used to identify the volatile organic compounds (VOCs) produced by PpR24 in a spray suspension and when applied *C. annuum* plants. Anti-microbial and plant-growth promoting VOCs were identified from the bacterial suspensions, whereas green leaf volatiles used in plant defence and signalling aphid natural enemies were identified from plants sprayed with PpR24. Significant differences were observed in the compound emissions of the biofilm-forming

isolate, PpR24b4, when compared to the wild-type PpR24. Non-target effects of PpR24 on three species of commercially available aphid natural enemies was investigated by simulating likely routes of exposure. PpR24 had no significant effect on the mirid bug, *Macrolophus pygmaeus* Rambur, although significant effects were observed on *Aphidius colemani* Viereck, and *Orius laevigatus* Fieber, depending on the route of exposure. The data presented in this study furthers our understanding of how PpR24 could be applied in an integrated pest management system to prevent and control aphid infestations.

Declaration

Declaration: I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.
Kristina Grenz

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Chapter 1

Literature Review

1.1 Introduction

The ever-increasing human population has put growing demands on the world's agricultural industry to become more efficient and increase crop yields. However, growers are faced with numerous challenges when attempting to cope with demand. A key issue is the impact of pest species.

Aphids are a global problem in horticulture and agriculture [1], with growers losing up to 10% of crop yields due to direct damage from aphid feeding and aphid-vectored diseases. Since the mid-1940s with the development of commercial synthetic chemical pesticides like DDT, chemical pesticides have been widely applied against insect pests, with over £5 billion spent on pesticides each year worldwide [2, 3]. Although still effective in many instances, increasing legislation and consumer concerns over the impact of chemical pesticides on the environment and human health has put more pressure on growers to find alternative green, biodiversity-friendly means of crop protection [4]. Chemical pesticides available to growers are limited further as many are dependent on the season, crop type and pest species. Target pests evolving resistance to treatments has also resulted in pesticide products becoming redundant. Intensive application of chemical pesticides has resulted in approximately 20 species of aphids evolving resistance to pesticides belonging to the main classes of chemical pesticides [5, 6]. Integrated Pest Management (IPM) strategies have become increasingly popular amongst growers in dealing with crop pests. By incorporating more specific natural enemies and biopesticides, combined with favourable crop culturing techniques, IPM offers a holistic approach to com-

bating pests rather than relying on synthetic chemicals that have a wider, and often more detrimental, impact [7](see Section 1.3.2).

This project, funded by the Agriculture and Horticulture Development Board, explores the potential of using aphicidal bacteria as biological control agents in sweet pepper glasshouse systems. Two previous PhD research projects discovered that the Gram-negative, rhizosphere-dwelling bacterium, *Pseudomonas poae* PpR24, possessed aphicidal properties when ingested by aphids. PpR24 was found to be able to survive on the leaf surface at no detriment to the host plant and was also found to deter aphids from colonising a plant [8, 9]. This study continues the investigation into the potential of PpR24 as a biocontrol, firstly by attempting to improve PpR24's efficacy as a biopesticide. The wild-type PpR24 is capable of killing 70% of aphids within 42 hours in a lab environment and can persist on a plant for three weeks. By employing experimental evolution, this project attempts to improve the virulence of PpR24 to aphids as well as its growth and survival on the plant. Further investigations will explore the volatile mechanisms behind the deterrent properties of PpR24 and whether it has undesirable non-target effects on natural enemies commonly applied to control aphids in an IPM system.

1.2 Aphids and their adaptations

Highly specialised to a phytophagic lifestyle, aphids are currently one of the most important pests to the agricultural, horticultural and forestry industries. There are approximately 4400 species of aphid, belonging to the family Aphidoidea, in the order Hemiptera [10, 11, 12]. Aphids inflict considerable damage on plants and are extremely hard to manage once an infestation takes hold, partly due to their complex life cycle.

Aphids are capable of reproducing sexually in the short-day winter months and asexually in the long-day summer months by parthenogenesis (Figure 1.1). This method of rapid, clonal reproduction allows devastating infestations to occur in relatively short periods of time. Most clones are wingless (apterous) females, but environmental stresses, such as starvation, over-crowding or the decline of the host plant's condition, may prompt the production of alate (winged) morphs (Figure 1.2). The winged phenotype has lower fecundity and spends longer in the pre-reproductive adult and nymph stages but allows for short and long distance dispersal, enabling an infestation to spread throughout

a crop [13].

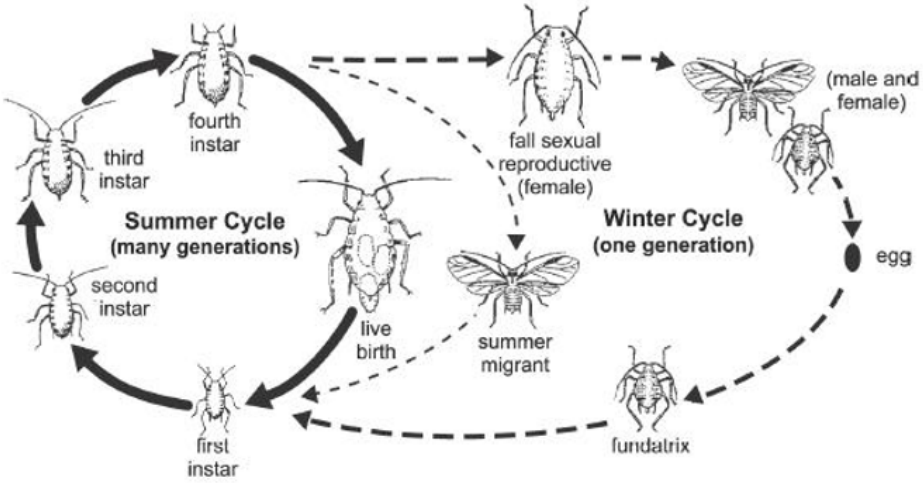


Figure 1.1: The cyclical parthenogenetic aphid lifecycle. During the long-day, summer months, aphids reproduce asexually by parthenogenesis. Over the winter months, aphids reproduce sexually and lay eggs which found the new colonies for the next year. Image taken from [14]

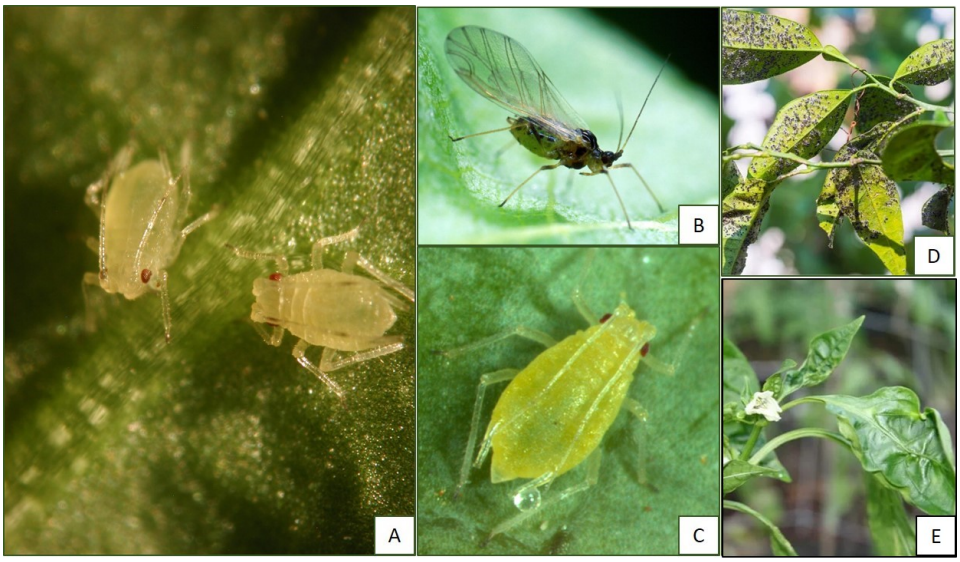


Figure 1.2: *Myzus persicae* appearance and effects on plants. A. *Myzus persicae* nymphs; B. alate morph *M. persicae*(Image by Scott Bauer [15]); C. apterous morph *M. persicae*(Image from [16]); D. sooty mold from excess honeydew produced by high levels on aphid infestation; E. leaf curling in sweet pepper caused by excessive feeding from aphids (Image from [17]).

Aphids form the largest group of insect phloem feeders [12] and their key adaptation is their plant-sucking mouthparts. Leaves are highly vascularized

and use tube structures, called sieve elements, to transport sugars produced by photosynthesis about the plant [18]. An aphid's maxillae and mandibles are elongated to form a stylet bundle that pierces the layers of plant tissue to reach the sieve elements while the labium assists by guiding the stylet from the outside [19, 10, 11]. During the feeding process, aphids secrete two types of saliva. The first is produced in the initial feeding stages and forms a protective sheath around the stylet bundle [11, 20]. By creating a path to the sieve elements, it reduces the chance of triggering the plant's defences during feeding. The second type of saliva is a watery form containing digestive enzymes [20]. This overcomes any clogging of the sieve elements in the plant's wound response, keeping the cells alive with a steady access to the phloem thus allowing the aphid to feed constantly for hours at the same site [21, 22, 23, 20]. Excess sugars are excreted from the aphid as a sticky honeydew [10] (Figure 1.3).

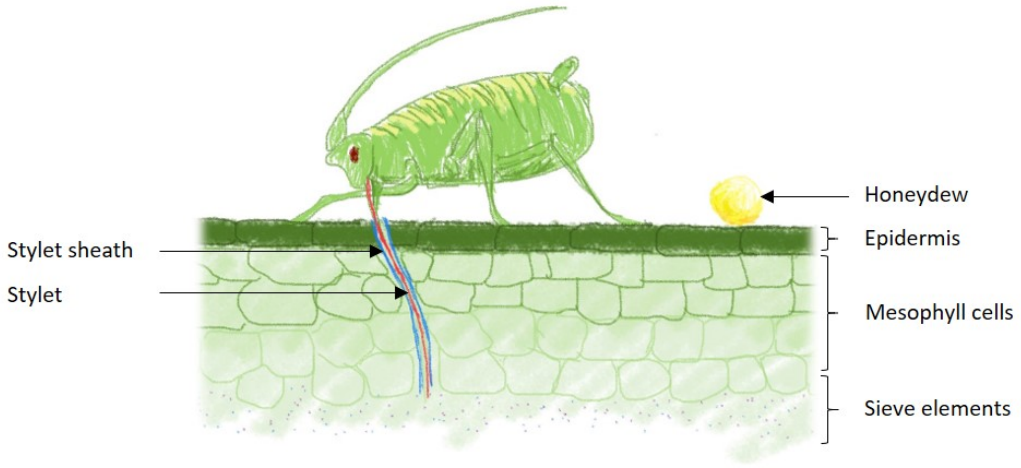


Figure 1.3: Aphid feeding apparatus. The stylet bundle pierces the plant epidermis and secreted saliva forms a protective stylet sheath, through the mesophyll cells to the sieve elements. Aphids feed off the sugars produced by photosynthesis that are transported through the sieve elements. Excess sugars are excreted from the aphid as honeydew.

Aphids are not regarded as a major threat to plants unless they occur in high numbers. Serious infestations cause significant economic damage to the agricultural industry every year from physical damage to crops through direct feeding, as well as being one of the most important arthropod vectors of plant viruses [24]. Although not as destructive as other insect pests, extensive feeding on plant phloem decreases growth rates and reduces crop yields. This can

also affect the aesthetic appearance of the plants, causing distorted growth in leaves and stems, such as twisting, curling or yellowing. Honeydew production can also be detrimental to plant growth. High in carbohydrates and nitrogenous compounds, excess amounts of honeydew encourages the growth of black mould. The mould can cause plant discolouration and reduce the amount of sunlight reaching the leaves, which can significantly affect photosynthesis levels in extreme cases [22, 25] (Figure 1.2).

The most significant damage to cultivated plants resulting from aphid activity is their ability to transmit plant viruses [12]. Aphids are responsible for 50% of insect vectored plant viruses [26], across various taxonomic groups, such as *Potyvirus*, *Carlavirus*, *Caulimovirus* and *Cucumovirus* [27]. The aphids' ability to disperse and reproduce rapidly, and the generalist feeding tendencies of some species, makes the spread of such viruses fundamentally hard to prevent. The annual cost of crops lost to aphid transmitted viruses may vary from year to year, depending on intensities of infestation, but viruses can prove devastating to crops. For example, cucumber mosaic virus, *Cucumovirus*, causes significant yield losses in a range of host plants and aphids are the primary vector [28].

1.3 Current methods of aphid control

1.3.1 Chemical controls

Chemical based pesticides and insecticides have significantly increased crop yields across the globe since their first use in the 1940s [29]. As an effective, immediate and affordable resource, they are an easy solution to dealing with serious infestations. However, increasing awareness and concern of the negative effects of insecticides on human health, the environment and the evolution of pest resistance to chemical pesticides from excessive applications has resulted in increasing restrictions on their usage [4].

The Insecticide Resistance Action Committee recognises over 25 modes of insecticide action, the majority of which target insect muscles and nerve pathways or affect insect growth and development [30]. The four classes of insecticide that dominate 70% of insecticide sales all act by interrupting nerve transmission [31]. Organophosphates and carbamate insecticides inhibit acetylcholinesterase, which plays an important role in the regulation of nerve

impulses. Pyrethroids interfere with volt-gated sodium channels and finally, neonicotinoids, such as imidacloprid, act by interrupting insect nicotinic acetylcholine receptors (nAChR) [32, 33, 31]. As insect muscle tissue has the highest density of nAChRs, paralysis and death can occur in a matter of hours [32, 33].

A major concern of the extensive use of pesticides is their impact on biodiversity and persistence in the environment. Once present they are hard to remove from an environment, and some pesticides can persist in soil for up to five months after a single application [34]. Run-off from treated fields has been found to pollute local water sources and act against non-target species [35]. The majority of synthetic pesticides act indiscriminately, often affecting non-target beneficial insects, reducing important ecosystem services, such as pollination, as well as disrupting the microbiota of the plant phyllosphere [36]. Numerous studies have focused on the decline of UK bee populations, particularly of honey and solitary bees, both important pollinators for the agricultural industry [37, 38, 39, 40, 41, 42].

Many factors are thought to contribute to the decline of bee species, including: climate change, diseases transmitted by the varroa mite and a reduction in bee-popular garden plants. The increase in pesticide and insecticide usage, particularly neonicotinoids, is also believed to in some part responsible for their dwindling numbers [34, 41, 32].

Acting systemically, the chemicals are absorbed by the plant tissue, thus providing extremely effective, long term protection against aphids and other plant feeding and phloem sucking insects. However, their systemic nature means that these chemicals spread to the nectar and pollen of plants, harming beneficial insects such as lepidopterans and bees [32, 34, 41]. Natural enemies are also significantly affected by pesticides. For instance, metaflumizone was shown to kill 80% of *Orius* and *Macrolophus* insects, two generalist predators commercially used to control crop pests. Chemical pesticides also have numerous sub-lethal effects on insect species, affecting reproduction, development and behaviour. Some cases have been seen to affect the physiology of insects. In the case of the brown plant hopper, alterations in the reproductive organs of the insects led to an increase in offspring which, coupled with the reduction of natural predators due to the pesticide, led to a more serious infestation [43].

Heavy dependence and overuse of chemicals has led to the evolution of resistance in some pest species. Pesticide resistance can evolve in several ways (for

a comprehensive review see [44]). A mutation may become selectively advantageous due to environmental change, such as the introduction of a pesticide, and resistant lineages spread throughout the population under the pesticide selection pressure. Pesticide resistance may also be occur in a pest population due to standing genetic variation already present in the pest population.

The most thoroughly documented instance of aphid-insecticide resistance is in the case of the peach-potato aphid, *Myzus persicae*, the most widespread and resistant species worldwide [5]. Chemical pesticides were used extensively against this species and, since the first reported instance of resistance in 1955, multiple forms of resistance have evolved against a range of chemicals, including organophosphates, carbamates and neonicotinoids [5, 45]. By overproducing carboxylases (E4 and FE4) that break down and sequester the insecticide esters, *M. persicae* can overcome the effects of many organophosphates, carbamates and pyrethroids [46, 47]. Mutations altering the insecticide target sites also confer effective resistance to applied pesticides. Modified acetylcholinesterase (MACE) can provide resitance to dimethyl carbamates and has been associated with an amino acid substitution in the active site of the enzyme, from serine to phenylalanine [48]. Mutations in volt-gated sodium channels have also been seen to grant effective resistance to pyrethroid pesticides, such as deltamethrin. Knockdown resistance (kdr), where leucine mutates to phenylalanine L1014F, and super-knockdown resistance, where methionine becomes threonine M918T, have also been observed in *M. persicae* [48, 49, 46]. In some cases, *M. persicae* individuals have been found to possess a combination of resistance mechanisms. This accumulation of resistance furthers the need to replace chemical pesiticides and insecticides with alternative methods of control, particularly the use of natural enemies [50].

In enclosed glasshouse cropping systems, concerns of chemical pesticide use generally stem from both risk of exposure to growers during application and the residues on produce that may affect consumers. However, with the use of beneficial insects in protected crops as pollinators and biological controls becoming more widespread, there are concerns about the compatibility of chemical pesticide use alongside such ecosystem-service providing insects. In some instances, chemical pesticides are advertised as suitable for use in IPM strategies due to the their high specificity to the target pest species. For instance, chlorantraniliprole, emamectin benzoate, indoxacarb and spinosad are said to be compatible for use in conjunction with the beneficial predator *Macrolophus*

pygmaeus to control pests. Thiacloprid is designed for use against hemipteran pests and as such is not suitable for use alongside some related natural enemies, for example *Orius* species. However, Broughton *et al.* [51] found that spinosad, though advertised in Australia for use as part of an IPM strategy, was harmful to commercial beneficials, such as mites, parasitoids and *Orius* bugs [32].

1.3.2 Integrated Pest Management and biocontrols

There is no ultimate strategy to combat all pests [52]. When used alone, many biocontrol agents and management practices are incapable of effectively reducing a pest below their economic damage threshold [53]. Therefore, they need to be used alongside other control measures in an integrated management system. Integrated Pest Management (IPM) is a holistic approach to pest control, employing a range of culturing methods and biological control practices to manage pests below their economic damage threshold, with forecasting to predict when pest outbreaks are likely to occur and only applying chemicals as a last resort. IPM strategies are becoming increasingly popular, especially in greenhouse protected edibles. For instance in the Netherlands, 90% of tomato, cucumber and sweet pepper produce is grown in an IPM system [54]. However, to be effective, growers require a sound understanding of the crop ecosystem to effectively manage the crop host, pest and control agent dynamics.

1.3.2.1 Cultural control

Growers can mitigate the impact of pests through various crop management practices and practical protection products, for instance, trapping pests with sticky traps, light traps and electric insect killers [55]. Ensuring good hygiene in the crop system, such as removing old pots and compost and weeding, is also vital in preventing pests from gaining a foothold in the crop. Intercropping with deterrent volatile producing plants, such as weeds, can actively deter pests from settling in a crop. Incorporating buffer zones around crops of non-crop plants can provide sink areas for pests to gather as well as a habitat for natural predators to establish [27, 56]. Attractive volatiles, such as insect semiochemicals used in mate location or plant volatiles, can also be used to create a push-pull system to lure pests to traps or buffer plant areas [57].

1.3.2.2 Biocontrol

There are three main approaches to biological control [58, 59]. Firstly, in cases of classical biocontrol, an invasive pest has established itself beyond its native range and natural enemies are introduced from their native habitat with the view to establish in the new system and provide long-term control of the invasive pest. One of the most well known examples of classical biocontrol was the accidental introduction to California of the cottony cushion scale, *Icerya purchasi*, in 1886. Originating from Australia, the cottony cushion scale devastated the Californian citrus industry until the introduction of the vedalia beetle, *Rodolia cardinalis*, from its native habitat which was able to establish in the environment and successfully control the pest [60]. Secondly, augmentative biological control involves the periodic release of natural enemies to maintain effective levels of pest control in glasshouse and open cropping systems. For instance, in Mexico 1.5 million hectares are treated with *Trichogramma* species, egg parasitoids to control lepidopteran pests [61]. Finally, conservation biocontrol focusses on nurturing the natural enemy populations already present in the environment by more indirect means, such as by planting attractive buffer crops to attract natural enemies [58].

1.3.2.3 Natural Enemies

More than 100 species of natural enemies of crop pests are currently commercially available worldwide [61]. In contrast to chemical pesticides, the management of crop pests by way of natural enemies provides the economic benefit of reducing yield loss without the negative environmental effects [62, 59]. Aphids have many natural predators and exploiting these natural enemies as part of an IPM strategy is one of the most pervasive methods of current pest control [63, 64, 65]. Common predators used include generalist predators, like the larvae of ladybirds, hoverflies, lacewings and gall midges, as well as adult spiders, carabids and rove beetles. Parasitic wasps are a popular form of aphid control as they act specifically to aphids [66, 67, 68, 69, 70, 71]. Prey frequency and prey density can significantly affect the success of introduced natural enemies. Due to their specificity, parasitic wasps will search out aphids even at low aphid densities, whereas more generalist predators may only be effective at high aphid prey densities. Many generalist species can also be phytophagous and can significantly impact a crop if not enough prey is found [52, 72, 59].

1.3.2.4 Biopesticides and entomopathogens

The term 'biopesticide' encompasses a range of biologically derived substances with various modes of action. Most biopesticides act selectively, leaving little to no residue on the crop, and can persist in the crop longer than many chemical pesticides, making them particularly attractive for use in IPM. Semiochemicals, such as insect sex pheromones, alter pest behaviour and can be employed in trapping systems or interfere with mate searching [54]. Plant derived biochemicals such as extracts, oils and secondary metabolites, can deter pest insects or attract beneficial ones [73].

The focus of this study is on microbial biopesticides. Entomopathogens, such as fungi, nematodes and bacteria, possess pathogenic properties and naturally play an important role in regulating arthropod populations. These virulent properties may occur for various reasons. For instance toxin production may promote bacterial establishment in an environment by inhibiting a host insect's immune system, disrupting host cell tissues and reducing microbial competition [74]. Several virulent species are exploited as biological controls as they present many advantages over both chemical and arthropod aphid management strategies [75]. Compared to chemical pesticides, entomopathogens are regarded as safer to use for humans and non-target organisms as they are generally highly specific. Furthermore, they have less of an environmental impact due to their biodegradeability and pose little or no threat to biodiversity [75]. Some entomopathogenic controls are also easier to store than insect biocontrol agents for long periods of time and can be applied to a crop using conventional equipment, such as foliar sprays and soil drench systems used to disseminate pesticides through crops [75]. However, biopesticides currently only account for approximately 5% of the global crop protection market and 90% of current products are derivatives of *Bacillus thuringiensis* [76, 77].

Bacillus thuringiensis (Bt) is a particularly virulent and effective form of insect control. The Gram-positive bacterium produces Cry proteins during its sporulation that activate in the gut when ingested by insects, breaking down tissue and rapidly killing the insect a few hours after ingestion [78, 75]. Due to its mode of action, Bt is highly specific to insects, posing no threat to birds, fish or mammals due to their more advanced, acidic digestive systems. Furthermore, as Bt must be ingested to be effective, it has less non-target effects than contact-acting alternatives. Although investigations into Bt's Cry-

related proteins have shown them to be effective against *Myzus persicae* [79, 80], Bt has failed to consistently control aphid infestations in the field [22]. The incorporation of Bt Cry toxins in transgenic crops has been used to create pest resistant cultivars, with the first successful case being Bt transgenic cotton in 1990 [81, 82]. However, although commercially available worldwide, legislation over the use of genetically modified organisms has limited the use of Bt crops in Europe [83].

Beauvaria bassiana is an entomopathogenic fungus increasingly used as a biocontrol agent due to its effectiveness against plant pathogens and arthropod pests [84]. One of its advantages when applied against insects is that it does not need to be ingested by the target. The fungus' conidium spores attach and enter through the chitinous cuticle where it germinates and feeds on the nutritional insect haemolymph below [85]. *B. bassiana* is effective against a range of pest insects, such as beetles [86, 87], lepidopterans [88, 89], grasshoppers and mites [90, 91].

The Enterobacteriaceae contain many phytopathogenic organisms that also have entomopathogenic potential. The bacterium *Dickeya dadantii* causes soft rot in important crop plants (such as potatoes, rice and maize [92]) but has also been found to be highly virulent when tested against the pea aphid, *Acyrthosiphon pisum* [79]. It possesses Cyt toxins similar to that of *B. thuringiensis*'s Cry toxins, which may explain its virulence. The Stewart's wilt pathogen, *Pantoea stewartii spp. stewartii* DC283, possesses aphicidal properties due to the *ucp1* gene that causes aggregations to form in the aphid gut, resulting in death within 72 hours [93].

Enterobacteriaceae residents of nematode guts have been shown to produce a range of bioactive compounds that have antimicrobial properties against bacteria, fungi and protozoa, as well as insecticidal functions [94]. The insect killing nematode, *Heterorhabditis bacteriophora*, is host to the insect pathogenic bacterium, *Photorhabdus luminescens* [95]. The nematode locates suitable prey, enters the haemocoel and proceeds to inject the bacterium into the victim [96]. Various toxins are produced, including insecticidal Toxin complexes (Tcs) and Makes caterpillars floppy (Mcf1) [95], resulting in insect death within 24 hours [97]. Toxin complex genes were first identified in *P. luminescens* strain W14 [98] where four toxin complexes; Tca, Tcb, Tcc and Tcd, were identified. Tc proteins comprise of only three genetic elements, tcaAB or tcdA-like genes, tcaC or tcdB-like genes and tccC-like genes. All three elements are required

for effective oral insecticidal toxicity [99]. The toxins act by disrupting the insect midgut epithelium, preventing further feeding and proving lethal to the host insect [100]. Similar *tc*-like *xpt* genes have been found in *Xenorhabdus nematophila*, another Gram-negative nematode-vectored pathogen with insecticidal properties, where the expression of three genes (*xptA*, *xptB* and *xptC*) is necessary for effective oral toxicity [101, 102, 103]. Homologues of *tc* genes have since been found in other insect associated bacteria, such as *Yersinia pestis* and *Serratia entomophila*, where *tc*-like genes are thought to be responsible for amber disease in the beetle grass grub *Costelytra zealandica*, a pasture pest endemic to New Zealand [104, 99]. There is scope for bacteria with Tc toxins to provide effective alternative biocontrol strategies to those that rely on Bt Cry toxins, such as in transgenic crops [100, 102].

1.3.2.5 Entomopathogenic pseudomonads

Pseudomonads are Gram-negative bacteria commonly found in the rhizosphere and phyllosphere of plants. They are a particularly promising class of bacteria to exploit as biological controls and extensive research has been conducted into their potential applications [105, 106, 107, 75]. Frequently found in agricultural soils, many Pseudomonads are plant growth-promoting rhizobacteria (PGPR) that enhance plant growth by making nutrients and compounds readily available to the plant as well as protecting their host plant from pathogens and pests [108, 109]. Fluorescent Pseudomonads in particular have been known to exhibit plant growth promoting properties. For instance, strains of *Pseudomonas fluorescens* have been found to have antimicrobial properties, suppressing fusarium wilt in tomato [110, 111] and the fungal disease, black root rot, in tobacco plants [112].

Members of the *Pseudomonas fluorescens* group have been seen to exhibit insecticidal properties [113]. In several species, virulence has been associated with the *fluorescens* insecticidal toxin (*fit*) gene cluster where *fitD* regulates the production of FitD, an important insecticidal toxin, which is found between *fitABC* and *fitE* genes that encode for a type 1 secretion system [114, 115]. Fit was found to be 73% similar to the Makes caterpillars floppy insecticidal toxin (Mcf1) produced by *Photorhabdus luminescens*, which acts by disrupting the insect midgut epithelium and hemocytes [116]. When present in *Pseudomonas protegens* CHA0, FitD confers lethal toxicity when injected into larvae of the

wax moth *Galleria mellonella*, and the tobacco hornworm *Manduca sexta*, an important agricultural pest [113]. FitD was also found to be important to the oral pathogenicity of *P. protegens* CHA0 and *Pseudomonas chlororaphis* PCL1391 to the diamondback moth *Plutella xylostella*, the African cotton leafworm *Spodoptera littoralis* and the tobacco budworm *Heliothis virescens*, all important lepidopteran pests [113, 115]. *Psuedomonas protegens* Pf-5 was also found to confer oral toxicity against the dipteran *Drosophila melanogaster* larvae [113]. However, pseudomonad insect toxicity is not solely dependent on FitD and in *fitD* deletion mutants, several strains retain some level of pathogenicity [114, 116].

Pseudomonas chlororaphis strains produce an array of metabolites that have antiomicrobial and insecticidal properties [117, 113]. Nematocidal properties have also been observed in *P. chlororaphis* O6, which is effective at controlling root knot nematodes, *Meloidoyne* spp., causing juvenile mortality and reducing gall formation. *P. chlororaphis* O6's toxicity is thought to be mainly due to the presence of *fitD* [113], however mutations in this gene did not affect the nematocidal properties of the bacterium. It is believed instead that the bacterium's virulent properties are due to the production of hydrogen cyanide [118]. Similar genes to *P. luminescens' tc* genes have been identified in pseudomonad strains, such as *Pseudomonas syringae* pv tomato and *Pseudomonas fluorescens* [119, 120, 113]. *Pseudomonas taiwanensis'* pathogenicity to a range of agricultural pests has been associated with the toxin complex component, TccC protein [121].

P. syringae PsyB728a, a bean pathogen, can kill pea aphids in 48 hours when administered via an artificial diet [122]. This virulence was associated with the *fliL* gene, the leading gene in the FliL operon which plays an important role in flagellum assembly and bacterial motility particularly swarming motility. *fliL*-induced swarming was hypothesised to regulate the expression PsyB728a-specific virulence factors that contributed to aphid death [122]. The aphids serve as a suitable secondary host to the bacterium, which is able to replicate in the aphid gut until it reaches lethal titers. However, as *P. syringae* is a plant pathogen, its application in a crop system is questionable.

Pseudomonads and other bacteria can be mass produced relatively easily due to their rapid reproduction cycle and their adaptability to a range of environments, thus there is scope for their use in a range of agricultural and horticultural settings [109]. However, Weller [109] and Kupferschmied *et al.* [123]

have voiced reservations on producing *Pseudomonas* sp. on a commercial scale as ensuring their survival during long-term storage will be challenging. Before any bacteria are used as treatment in the field they must undergo rigorous trials to ensure there are no deleterious effects on other species or risk to human health and the environment [123]. Furthermore, it has been found in some endophytic bacteria that ordinarily mutualistic organisms turn pathogenic under stressful environmental conditions [124, 125]. However, as living organisms bacteria applied as biopesticides have the potential to adapt and evolve along with aphid pests, thus reducing the likelihood of aphids evolving resistance to the management method.

1.4 My project

1.4.1 Previous research

This project is a continuation of the PhD research conducted in the Jackson Lab by Dr Amanda Hamilton and Dr Deepa Paliwal, investigating the potential phylloplane biocontrol agent *Pseudomonas poae* PpR24. PpR24 is a Gram-negative, rod-shaped bacterium, capable of movement by a single polar flagellum [126]. PpR24 was first isolated from cabbage roots during Dr Hamiltons' research, where bioprospecting was applied in a bid to discover potential biopesticides for use against aphids and thrips [8]. Out of 140 bacteria isolated from the rhizosphere and phylloplane of a variety of plant species, three were found to be most pathogenic to aphids. *Pseudomonas flourescens*, *Citrobacter werkmani* and *Pseudomonas poae* were effective at killing six species of aphid: *Myzus persicae*, *Brevicoryne brassicae*, *Aphis fabae*, *Macrosiphum albifrons*, *Aulacorthum solani* and *Nasonovia ribsnigri*, with 100% mortality observed after 72 hours when applied at 10^7CFU ml^{-1}. Dr Hamilton observed no adverse hypersensitive response to the bacteria in the experimental plants or in five non-target insect species tested (which shall be discussed further in Chapter 6), and also found evidence of plant growth promoting properties.

The follow-up study conducted by Dr Paliwal [9], concluded that out of the isolates under scrutiny, *Pseudomonas poae* PpR24 was the most promising as a potential biocontrol agent. In addition to its efficacy against the aphid species mentioned above, further virulence investigations found *P. poae* to be effective at killing four aphid clones known to be resistant to numerous pesticides,

with 50-80% aphid mortality when applied at bacteria dose of 10^7CFU ml^{-1}. Furthermore, PpR24 was able to survive on *Arabidopsis thaliana*, *Capsicum annuum* and *Beta vulgaris* plants for 21 days when applied as a foliar spray, with no adverse hypersensitive response. For each plant species tested, significantly fewer aphids were present on plants inoculated with *P. poae*, suggesting PpR24 had an aphid deterrent effect. This deterrence was further supported by olfactometer assays, where alate aphids exposed to volatiles extracted from a plant inoculated with *P. poae* exhibited a deterrent or repellent response. However, this deterrent effect was not observed when aphids were exposed to volatiles extracted from *P. poae* alone.

To elucidate PpR24's aphid-killing mechanisms, Paliwal employed transcriptome profiling by RNA-seq and knock-out mutagenesis to identify virulence factors. Transcriptome profiling by RNA-seq found transcriptomal changes in the regulation of 193 genes in *Myzus persicae* and 1325 genes in *P. poae* PpR24 over the course of infection. As well as stress and virulent genes, many genes modulated during infection were associated with metabolic processes. Paliwal found that the *P. poae* genome contained genes associated with insecticidal toxins. Although *fitD* was not present, two Rearrangement hotspot (Rhs) proteins and two metalloproteinases (AprA and AprX) were identified with known insecticidal functions combating host immune response [121], as well as genes encoding for proteins belonging to the Tc insecticidal toxin complex: two TcA-like (TcaA1 and TcaB1), one TcB-like (TcaC1) and one TcC-like (TccC2) insecticidal toxins. Due to the presence of these genes, it was hypothesised that *P. poae* may have a similar mode of action to Tc possessing pathogens like *P. luminescens* and disrupt the midgut tissue of the host insect. Further analysis by knock-out mutagenesis of selected toxin genes resulted in no complete reduction in aphid mortality, suggesting a combination of genes contributed to aphid pathogenicity. However, the absence of the metalloprotease *aprX* gene saw the most significant reduction in aphid mortality, with only a 20% aphid mortality rate in 48 hours. Furthermore, as *P. poae* was able to replicate inside the aphid gut, it was hypothesised that bacterial occlusion in the aphid gut, similar to that observed in *P. syringae* PsyB728a [122], may also contribute to aphid virulence by preventing the aphids from feeding.

Overall, PpR24 shows a great deal of promise for future use as a biopesticide product it terms of persistence on a crop plant and efficacy at managing aphid

populations. This project is a continuation of the investigations into PpR24's suitability as a biocontrol.

1.4.2 The model system

This study will focus on the affects of *Pseudomoans poae* PpR24 against the aphid *Myzus persicae* when applied as a foliar spray in sweet pepper crops, *Capsicum annuum*. Over 1.6 million tons of pepper fruit is grown annually across the world. High in calcium and vitamins A and C, it is a popular crop. In the UK pepper is an important protected edible, with 90 hectares of pepper grown in controlled hydroponic glasshouses (as of 2015) [127]. Although various pests afflict pepper crops, the primary factor that leads to insecticide use is the need for aphid control. The green peach potato aphid, *Myzus persicae*, in particular is a global pest of sweet pepper but also has a wide host range of approximately 400 plant species [5] and acts as a vector for over 100 plant viruses. *M. persicae* is renown for developing resistance to insecticides [5] therefore novel, effective forms of control are needed.

Anecdotally, visiting Tangmere Airfield Nurseries Ltd, the largest commercial sweet pepper grower in the UK, and other growers across the UK highlighted the strong desire of many growers to reduce chemical pesticide use in favour of biological control alternatives. Important considerations growers face when using a new biopesticide is whether it is cost-effective and compatible with the current systems in place in the glass house, such as spray systems or drenching. In the case of pepper plants the challenge of monitoring and delivering effective pest control became apparent not only due to the vast size of the nursery (75 acres of crops under glass) but also due to the height of the crops as mature pepper plants in their raised hydroponic beds can reach at least two metres high, a significant challenge for effective pest monitoring (Figure 1.4). An example of other glasshouse environments, Tangmere employs an IPM system to control aphid and other pests, as well as using insects to pollinate the crops. A potential biopesticide would have to function alongside such service providing insects. Although many protected edibles and ornamentals are frequently sprayed to prevent pests, Tangmere growers emphasised that they minimised spraying their peppers as much as possible to prevent plant disturbance which would divert energy from fruit production. Ideally any biopesticide would be applied as a soil drench or as a seed-coating.

However, were the success rate of a biopesticide nearer 100% pest mortality, or the biopesticides persistence on the plant durable enough to merit minimal spray applications, biopesticide sprays to treat crops would be considered.

Figure 1.4: Tangmere Airfield Nurseries Ltd. Environment controlled glasshouses each house approximately 290000 pepper plants (A). The plants are grown in raised hydroponic beds (B) for ease of waste matter management.

1.4.3 Research aims and objectives

The overall purpose of this project was to further the current understanding of *Pseudomonas poae* PpR24's potential as a biocontrol agent. The aims for each research chapter can be more precisely defined as follows:

1. Chapter 3 employs an experimental evolution approach in a bid to improve PpR24's fitness inside the aphid and on the plant phylloplane without directly modifying PpR24's genome. Serially passaging PpR24 through the aphid gut via artificial diet sachets was conducted in a bid to improve PpR24's virulent properties, as well as serial passaging of PpR24 in broth microcosms in a bid to evolve a biofilm phenotype on the hypothesis that biofilm formation may improve bacterial fitness on

a plant and potentially aid bacterial virulence by assisting gut occlusion. An investigation into whether any trade-offs occurred between the evolved isolates and the wild-type PpR24 was also conducted.

2. To elucidate phenotypic differences between PpR24 isolates evolved in Chapter 3, Chapter 4 applies whole genome sequencing and variant calling analysis to identify mutations present at the conclusion of the serial evolution passages that differ from the wild-type PpR24.

3. Chapter 5 explores PpR24's aphid-deterrent effect observed in the Paliwal study. An investigation into the volatile emissions of the wild-type and derived PpR24 isolates intended to identify aphid-deterrent volatiles produced by the bacteria when in a broth and on a plant environment. Whether derived isolates of PpR24 from Chapter 3 are more effective at deterring *Myzus persicae* from crop plants than the wild-type PpR24 shall also be investigated.

4. Direct non-target effects of PpR24 on natural enemies commonly used to manage aphid glasshouse infestations is explored in Chapter 6. Experiments simulating likely routes of exposure natural enemies may encounter a foliar sprayed biopesticide shall be conducted using the parasitic wasp, *Aphidius colemani*, the hemipteran *Orius* bug, *Orius laevigatus*, and the green mirid bug,*Macrolophus pygmaeus*.

Chapter 2

General Materials and Methods

2.1 Media

All bacteria, unless otherwise stated, were grown in Kings Medium B Broth (KB), at 27°C shaken at 200rpm or on a KB agar plate [128] at 27°C. Proteose peptone 20g, K_2HPO_4 1.5g, $MgSO_4.7H_2O$ 1.5g and glycerol 10ml were added to 1L of deionised water. Agar was added to the media to achieve a concentration of 1.5%. Media were autoclaved at 121°C for for 20 minutes for sterilisation. Sterile KB agar was melted and 20ml poured into 9cm diameter Petri dishes (Thermo Fisher Scientific, Scotland, UK) when cooled to 50°C. For PBS used throughout the project, 1L of 10 x PBS contains 80g NaCl, 2g KH_2PO_4, 29g $Na_2HPO_4.12H_2O$, 2g KCl; 1xPBS has a pH of 7.4.

2.2 Bacteria

Pseudomonas poae (*P. poae*) PpR24 was originally isolated in the University of Reading Experimental Greenhouse from *Brassica oleracea* roots by Hamilton (2016) and was found to be Ampicillin and Nitrofurantoin resistant [9]. Frozen stocks were made using 500μl of 50% glycerol solution and 500μl bacterial-1xPBS suspension and kept at -80°C.

2.3 Plants

The plants used throughout the project were sweet pepper Palermo RZ F1-Hybrid *Capsicum annuum* L., supplied by Rijk Zwaan seeds. The plants were

grown at 21°C at 70% humidity in a controlled environment room on a long day light cycle (16hr light/8hr dark) for four weeks before use in experiments or for rearing aphids.

2.4 Aphid rearing

All aphids used were *Myzus persicae* Sulzer (Hemiptera: Aphididae) supplied by Rothamsted Research. Clones were maintained parthenogenetically in plastic leaf box cages or on whole plants if large populations were needed. The insects were kept in a rearing room at 21°C on a long day light cycle (16hr light/8hr dark) to ensure no sexual reproduction occurred.

2.5 Aphid mortality sachets

All aphid mortality assays were conducted in a constant environment, long-day light cycle rearing room at 21°C and 70% humidity. Preparation of the sachets was carried out in a laminar flow hood to mitigate contamination.

The aphid Mittler diet was used in feeding sachets [129]. Ingredients were added to 100ml of water with 15g of dissolved sucrose in the order and quantities as laid out in Table 2.1 in Appendix A, ensuring each compound was fully dissolved before the addition of the next. The solution was then stored at -20°C in 50ml falcon tubes.

Perspex cylinders, 25mm in diameter by 25mm in length, were cleaned with 70% ethanol and one end covered with 4cm^2 of parafilm. Two other sections of parafilm were cut and all were placed under UV light for sterilisation in a safety cabinet for 35 minutes.

Room temperature, pre-prepared Mittler diet was sterilised with a 0.22μm filter syringe. Control sachets were made up of 594μl diet and 6μl sterile water, which was pipetted onto the sterile parafilm stretched over the top of the cylinder and carefully covered with a second layer of sterilised parafilm to avoid any spillage (Figure 2.1).

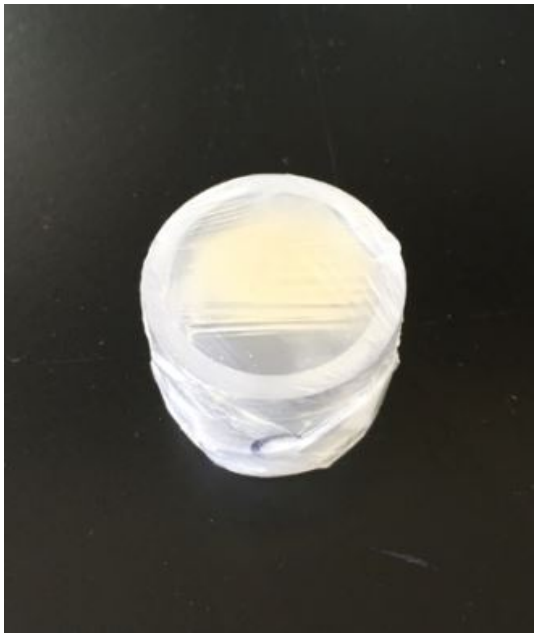


Figure 2.1: Aphid diet sachet. Sachets were comprised of 25mm by 25mm perspex cylinders, with one end covered with 4cm ^{2} of parafilm. 594μl of Mittler diet with either 6μl sterile water or bacterial-broth suspension was pipetted onto the parafilm and covered with a second layer of parafilm to make the sachet. Ten aphids were added to the cylinder and sealed in with another layer of parafilm.

'Treated' sachets were prepared in the same way. *Pseudomonas poae* strains were recovered from stocks kept at -80°C and grown for 24 hours on KA plates at 27°C to achieve single colonies. A single colony was picked and the bacteria were then grown overnight, shaken at 27°C in 10ml KB media for 16 hours. A spectrophotometer was used to analyse the bacterial cell density and normalised to an OD600 to 1, which was equivalent to a bacterial concentration of 10^9 CFU ml^{-1}. After washing in sterile 1xPBS, 6μl of bacterial suspension was added to 594μl of Mittler to achieve a concentration of 10^7 CFU ml^{-1} and this formed the sachet filling.

Ten aphids were delicately added to each cylinder using a paintbrush and the cylinder sealed with the final piece of parafilm. The cylinder was placed with the sachet at the top so any dead aphids can drop to the bottom and easily recorded. Observations were made at 0, 1, 6, 18, 24, 30, 42, 46 and 48 hours.

2.6 Bacterial foliar spray

Plants were grown as described in Section 2.3. *Pseudomonas poae* strains were recovered and grown from frozen stocks as mentioned in Section 2.5. Bacteria were washed twice in 1xPBS to remove any residual growth media and resuspended in 1xPBS at an OD600 to 1 to form the spray suspension. Plants were sprayed with 8ml of suspension (unless otherwise stated) on the abaxial and adaxial leaf surfaces using a hand atomiser spray nozzle at a cellular suspension of 10^7 CFU mL^{-1}. Sterile 1xPBS was used as a control spray. Nozzles were cleaned with 70% ethanol and sterilised water before use, with each spray treatment using a different nozzle to prevent contamination. After spraying, plants were left in the laminar flow until completely dry.

2.7 DNA extraction

DNA was extracted and purified using a Qiagen Puregene Core A kit following the protocol guidelines for Gram-negative bacteria. A sample was electrophoresed in a 0.8% agarose TBE gel to check for DNA integrity and the concentration was measured using a NanoDropND-1000 UV-Vis Spectrophotometer. Extracted DNA was stored at 20°C.

Monarch PCR and DNA cleanup kit was used, following the protocol for dsDNA $<$ 2kb and the samples were sent to Eurofins for Sanger Sequencing.

2.8 Polymerase Chain Reaction (PCR)

PCRs were carried out using PCRBIO Taq Mix Red (PCR Biosystems Ltd, London, U.K.), following the protocol of 2x PCRBIO Taq Mix Red, 1μL of each 10μM forward and reverse primer, 1-2μL template and molecular biology grade water to 25μL.

The initial denaturation was at 95°C for two minutes, followed by 30 cycles of denaturation at 95°C for one minute and annealing at 50-65°C for 20 seconds to 1 minute (depending on the primer pair used). Extension was at 72°C at one minute kb^{-1} and a final extension step of 72°C for five minutes.

2.9 Phusion PCR

Phusion high fidelity DNA polymerase (Thermo Fisher Scientific, Scotland, and U.K.) buffers and enzyme were used for gene sequencing. The reaction was prepared as follows: 10μL 5x Phusion HF buffer; 1μL 10mM dNTPS; 1μL of each 10μL forward and reverse primer; 0.5 1μL template; 0.5μL Phusion polymerase (1 unit/50μL); molecular biology water to 50μL. Cycling conditions were as follows: initial denaturation of 98°C for 30 seconds; 30 cycles of 98°C for ten seconds, 58°C for 30 seconds, 72°C at 30 seconds kb-1; and a final extension of 72°C for ten minutes. Primers were designed using Primer3Plus and checked for their specificity using NCBI Primer-BLAST.

Table 2.1: Primers used throughout this study.

Target	**Primer name**	**Sequence (5'->3')**	**Temp. °C**	**Product size (bp)**	**Function**
tcaA toxin	TcaA1poae_F1	TAAGGATTACACCGGCCAAC	58	524	*P. poae* diagnostic primers
	TcaA1poae_R1	TTTCTTTCAACGGCTGCATT	58		
cheB	cheB_F1	GACTTGATCATGCCGGTGAT	57	564	Amplification of *cheB* gene
	cheB_R1	TAGGCTAGCGTGCCATTTTT	55		
barA	bar_F1	CGCTATTTACTGCCGGTGTT	57	433	Amplification of *barA* gene
	bar_R1	TCTGGATCTCGATGGTTTCC	61		

2.10 Agarose gel electrophoresis

Gels were cast using Bioline Molecular Grade Agarose powder. Dependent on the required final concentration (1-1.5% w/v), agarose powder was dissolved in 0.5X AmbionTBE buffer (10x solution contains 0.89 M Tris, 0.89 M Borate, 0.02 M EDTA). Biotium Gel Red(10,000X in water) was added to a final concentration of 0.1 mg mL^{-1}. 10X DNA sample buffer (200 mM Tris-HCl, 5 mM EDTA, 30% (v/v) Glycerol, 0.1% (w/v in water) Bromophenol blue, 0.1% (w/v in water) Xylene cyanol) was added to DNA samples to a final concentration of 1X and the samples loaded and run in a BIORAD gel tank at a voltage of 120 mV for the desired amount of time (usually 45 minutes to 1 hour). BIOLINE HyperLadder1 was run in tandem with the samples as a DNA band size marker. On completion of the run, DNA bands were visualized and photographed in a G:box (Syngene).

Chapter 3

Experimental evolution to improve *Pseudomonas poae* PpR24 as a biocontrol

3.1 Introduction

Due to the increase in pest resistance and environmental pressure to move away from chemical pesticides, new forms of aphid biological control are in high demand. Previous work [8, 9] demonstrated the potential of the rhizosphere bacterium *Pseudomonas poae* PpR24 as an aphid biocontrol agent. The bacterium is effective at suppressing six species of aphid, reducing aphid populations by 70% in 48 hours at no detriment to the host plant when applied as a foliar spray, soil drench or leaf innoculation [8, 9]. *P. poae* PpR24 was also shown to survive on the plant and remain effective for 21 days. Although already successful at controlling aphids, this chapter explores whether the aphicidal virulence of PpR24 can be enhanced and whether PpR24's survival on the plant phylloplane can be improved using an experimental evolution approach.

3.1.1 Experimental evolution

Since its first use by Dallinger in 1878 investigating the heat tolerance of 'septic' organisms [130], and championed in modern times by Lenski's seminal long-term evolution project with *Escherichia coli* in the 1980s [131], experimental evolution has become a well-established method for examining the underlying mechanisms of evolution, such as natural selection and genetic drift.

By passaging an 'ancestor' organism in a controlled selective environment for multiple generations, adaptations resulting from random mutations and standing genetic variation in the population can arise and the divergent populations can be tracked and observed by experimenters in real time [132]. Unlike artificial selection, where experimenters select for the desirable traits they wish to evolve, experimental evolution involves only the conditions of the experimental environment being dictated by the experimenter. Consequently, the population under study are at liberty to adapt and evolve traits to increase their fitness in the environment without guidance [133].

Experimental evolution has been applied extensively in microbial studies [134] as micro-organisms are particularly well suited to such studies due to their rapid generation time and small genome sizes that allow mutations to occur and establish quickly in a population. The relatively low cost of whole genome sequencing means that it is fairly accessible for studies to trace and identify mutations [135, 136, 132]. Furthermore, microbes are able to be stored in a non-evolving state as frozen stocks, enabling a 'fossil record' of each passage to be kept for analysis at a later date [131].

In addition to being very informative from a pure science perspective and for investigations of evolutionary theories, several studies have applied experimental evolution to better understand the underlying mechanisms of human pathogen adaptation and antibiotic resistance, as well as develop novel forms of bacterial infection treatment [137, 138, 139, 140, 141, 142]. For example, Jochumsen *et al.* [138] investigated the causes of colistin-resistant *Pseudomonas aeruginosa* isolates in cystic fibrosis patients. Sixteen lineages of *P. aeruginosa* PAO1 were serially passaged for 62 cycles with increasing concentrations of colistin, resulting in nine lineages evolving high-level colistin antibiotic resistance. Colistin resistance was found to be a multi-step process, requiring at least five mutations at independent loci, especially in pathways featuring *pmrB*, which regulates lipopolysaccharide modification in the outer-membrane, and *opr86*, involved in outer-membrane protein assembly.

Experimental evolution also provides an environment to develop novel medicinal treatments and test how resistance may evolve in target pathogens, for instance in developing new drug or phage cocktails [139]. Scanlan *et al.* [139] applied experimental evolution to phage therapy, a potential novel approach to treat bacterial infections. Pathogenic bacteria can evolve resistance to phage therefore experimental evolution was applied to investigate how this

resistance evolves and whether any costs to the bacteria are associated with it, such as slower growth or less virulence to the host, which may be insightful in developing more effective treatment strategies.

3.1.2 Experimental evolution in agriculture

There is scope to apply experimental evolution to a range of topics in an agricultural setting and there is growing interest in how experimental evolution can address key issues in crop protection [143, 144]. Growers are continually seeking novel methods to increase crop yields and experimental evolution may offer the opportunity for enhanced biological-based products without the negative connotations often associated with genetically engineered products by the public.

Experimental evolution has been applied to attempt to evolve improved plant-growth-promotion in bacteria [145, 146]. Nitrogen fixation is a key process by which root associated bacteria, rhizobia, can fix nitrogen for plant growth. Many species of rhizobacteria have a symbiotic relationship with legume crops and colonise plant roots intracellularly by root nodule formations. To better understand how the symbiotic relationship between bacteria and legume crops occur and whether nitrogen fixation can be enhanced to boost plant growth, experimental evolution and genome resequencing was applied to combine the root-infecting wilt pathogen *Ralstonia solanacearum* and the nitrogen-fixing symbiont *Cupriavidus taiwanensis*. After inserting the symbiotic plasmid of *C. taiwanensis* into *R. solanacearum*, phenotyping following 16 selection passage cycles showed the successful evolution of several symbiotic traits and a reduced plant immune response to the formation of root nodules, indicating improved symbiosis and potential for improving beneficial bacteria symbiosis by experimental evolution in future. However, no improvement to nitrogen fixation was observed likely due to the low number of passage cycles [145, 146].

Experimental evolution is also a powerful tool in understanding factors that drive adaptation and the evolution of resistance in pest pathogens and insects to pesticide treatments and resistant cultivars. *Ralstonia solanacearum* has a wide host plant range and experimental evolution coupled with whole genome sequencing was applied to unravel the underlying factors in host plant adaptation. After 300 bacterial generations of serial passaging on either an orig-

inal or distantly related host plant, the majority of derived *R. solanacearum* clones had improved fitness on the plant regardless of the host plant. However, fitness gains were most significant on plants more distantly related to *R. solanacearum*'s usual host plants [147]. Such insight into the flexible adaptability of *R. solanacearum* could be important to growers in terms of designing disease management plans, such as cultural control practices involving crop rotation and intercropping with less susceptible plants [148]. The selective passaging method has also been used to investigate the potential for the greater wax moth, *Galleria mellonella*, to evolve resistance to the biopesticide *Bacillus thuringiensis* (Bt). After 30 generations, *G. mellonella* larvae evolved a 10.8 fold increase in resistance to Bt [149].

Whether non-target species can evolve to cope with pesticide exposure has also been investigated with an experimental evolution approach. The water flea, *Daphnia magna*, is a common species in water bodies of agricultural landscapes at risk of being exposed to pesticide run-off and is a suitable model organism. *D. magna*'s cyclic parthenogenic reproduction allows for clonal populations to establish rapidly and the production of dormant eggs allow for gene pool samples to be collected with relative ease. After experimental passages exposed to the pesticide carbaryl, the majority of descendant *D. magna* populations had evolved a tolerance to carbaryl [150, 151].

An important issue in crop protection addressed by existing experimental evolution studies is understanding how herbicide resistance evolves in weeds. By better understanding how herbicide resistance occurs, growers may be able to develop more effective application strategies and crop management to prevent resistance from occurring [152, 153]. For instance, experimental evolution has been applied to investigate how quickly plants evolve herbicide resistance under different application strategies [154, 155]. When cycling the use of the herbicides atrazine, glyphosate or carbetamide, some cycling combinations were found to prevent herbicide resistance evolution [156]. Exposing plants to a mix of herbicides concurrently also resulted in reduced resistance evolution when applied in their advised doses [156].

However, there are practical limitations when applying experimental evolution to a crop set up. Due to their relatively long life cycle, the difficulty in maintaining large populations for prolonged periods of time, and the small number of generations, plant-based studies often suffer from a limited number of passages, therefore the direct relevance of studies to field applications is

questionable [154]. On the other hand, new developments in speed breeding for experimental procedures may make experimental evolution studies more feasible in certain crops. By drying seeds and extending the photoperiod plants are exposed to, crop production in wheat, barley, rapeseed and chickpea was seen to increase from 2-3 to 4-6 generations in a year [157].

3.1.3 Aims of this Chapter

3.1.3.1 Evolving improved aphicidal properties

Of particular relevance to this study is the potential for experimental evolution to improve existing beneficial microbes without the stigma of producing a genetically engineered product. Commercial producers of biopesticides continuously seek for products with traits as close to the efficacy of chemical pesticides as possible, such as rapid pest mortality, a wide target range with minimal non-target effects, and to be successful in a variety of environmental conditions. Studies have applied experimental evolution in attempts to improve the performance of already effective biopesticides. For example, experimental evolution was applied to the entomopathogenic fungus, *Metarhizium anisopliae*, to improve its performance as a biological control against insect pests. *M. anisopliae* has a wide host range but is sensitive to heat stress, with little to no growth between 35-37°C, thus limiting its use for growers. Upon infection of target pests, the insect host can enter a state of behavioural fever and inhibit conidial germination of the fungus, preventing effective control. Serial passaging of *M. anisopliae* resulted in improved survival of the fungus at previously uninhabitable temperatures [158]. Another investigation used experimental evolution in a bid to improve virulence of *Beauvaria bassiana* isolates Bb1520 and Bb8028 to malaria mosquitoes, *Anopheles coluzzii*. However, after 10 passages no significant improvement in *B. bassiana* virulence was observed, although derived lineages were able to successfully colonise previously uninhabitable growth substrates [159].

The primary aim of this chapter was to investigate whether the aphicidal properties of *Pseudomonas poae* PpR24 could be improved by experimental evolution. To evolve improved aphid killing, PpR24 was serially passaged through aphids by means of inoculated diet for ten cycles. In an applied setting, this may reduce the time taken to combat aphid infestations on the crop and decrease the transmission of aphid-vectored plant viruses.

3.1.3.2 Biofilms and crop protection

Secondly, the wild-type PpR24 was serially passaged in an attempt to evolve biofilm formation. Biofilms are aggregations of microbial cells that form on biotic or abiotic surfaces. Biofilms are held together by the secretion of extracellular polymeric substances (EPS), for instance exopolysaccharides and polypeptides [160, 161], and cells cooperate and communicate with each other by methods such as quorum sensing [162, 163]. There are many advantages to living in a biofilm community that can improve bacterial fitness in a niche, for example ease of sharing resources, gene transfer and in some cases antibiotic resistance. Biofilms can play a significant role in infectious diseases and human pathogens. *Pseudomonas aeruginosa* is one well studied pathogen and often its ability to form biofilms inhibits successful antibiotic treatments, resulting in chronic infections [163, 160].

Many biofilms are associated with plants and have been found to colonise plant stems, roots and leaf surfaces (phylloplane) [164, 165], as well as internal surfaces. The plant pathogen *Xyllela fastidiosa* is the cause of Pierce's disease in a variety of important crop plants, such as grape vines, peach and citrus plants. Infected crops wilt and defoliate prematurely, partly due to biofilm aggregates restricting water flow in the xylem vessels of the plant host [166]. Biofilms are also suspected to play a key role in infection of the wilt disease pathogen, *Ralstonia solanacearum* on its tomato host [164]. Biofilm aggregations on tomato seedling roots and in the plant xylem are thought to aid *R. solanacearum* establishment [167]. On the phylloplane, bacterial biofilms are most often found in the nooks and crannies of the leaf surface, veins and the base of trichromes. For instance, *Pseudomonas syringae* B728a was observed preferentially forming aggregations at the base of trichromes, possibly due to the gathering of water droplets at their base [164]. The phylloplane can be a harsh environment for bacteria, with challenges such as UV radiation, nutrient limitations and competition from other microbes. Thus, the biofilm lifestyle provides a means of surviving under such stresses, offering protection from the elements and rival micro-organisms. Another factor to consider in terms of potential benefit to a biocontrol agent, is that an evolved proficiency to form biofilms may improve bacterial occlusion in the insect gut and consequently kill host insects faster [9, 122].

There is potential to recruit biofilm forming bacteria and fungi in a variety

of roles to improve crop yields, for instance as biofertilisers and plant growth promoters [168, 169]. Of most relevance to this study is evidence that biofilms have biocontrol properties [169]. Biofilm formation in the bacterium, *Pseudomonas putida* A1, may assist in its antimicrobial activity against several plant soil pathogens, including *Ralstonia solanacearum*, by adhering to root wound sites and inhibiting pathogen growth [170]. *Bacillus subtilis* biofilm colonisation on plant roots has also demonstrated the potential for crop protection [169]. *B. subtilis* 6051 was demonstrated to reduce arabidopsis susceptibility to the pathogen *Pseudomonas syringae* by forming an anti-microbial producing biofilm [171].

When in static (heterogeneous) and shaken (homogenous) broth suspension, the wild-type PpR24 naturally assumes a free-floating planktonic state and does not exhibit biofilm formation. However, by applying an experimental evolution approach and serially passaging PpR24 in a selective environment, biofilm formation may evolve. Precedent for such an experimental approach has been establish in the related *Pseudomonas fluorescens* SBW25 Wrinkly Spreader (WS) mutant [172, 173, 174, 175, 176, 177, 178]. Similar to PpR24, the soil-dwelling wild-type *Pseudomonas fluorescens* SBW25 does not naturally assume a biofilm life-style when grown in broth. However, serial passages of SBW25 through microcosms of KB broth resulted in the adaptive evolution of strong biofilm forming mutants at the air-liquid interface. These adaptive mutants generate a Wrinkly Spreader genotype (WS) as the result of a single point mutation in *wsp*, a chemosensory-like regulatory apparatus responsible for cellular attachment and cellulose expression. The combined cellulose matrix and fimbrial attachment structure forms robust biofilms. The WS phenotype grants the mutants a fitness advantage in the microcosm environment, enabling them to maximise oxygen uptake at the oxygen-rich broth surface, grow faster, and out-compete non-mutant bacteria in the microcosm.

By employing a similar methodology to that of the Wrinkly Spreader experiments, an investigation into whether it was possible to evolve biofilm formation *in vitro* in derived PpR24 isolates was carried out. Such an adaptation may enhance PpR24's colonisation and survival on host plants, as well as potentially improve the biocontrol properties of PpR24 if gut occlusion is an important aspect of the aphid-killing trait. Therefore biofilm formation may be a significant beneficial property to a biopesticide agent.

3.1.3.3 Trade-offs between evolved isolates

The final objective of this chapter was to assess whether any adaptive trade-offs occurred between the wild-type and evolved PpR24 isolates as a result of the two selection regimes, or indeed, whether evolved traits compliment and reinforced each other.

Adaptation can result in trade-offs between traits, where one trait comes at the cost of another [179, 140]. For instance, in the case of the SBW25 WS genotype, although the WS mutation provided a fitness advantage in static KB microcosms, mutants performed less well on agar plates and in shaken environments, indicating a trade-off between biofilm formation and the ability to survive in these environments [172]. The performance of the evolved PpR24 isolates in the alternative selective regimes was examined and an investigation into trade-offs in traits known to be associated with biofilm formation and bacterial virulence conducted. In addition to comparing the success of the final-passage isolates, evolved isolates were compared to the wild-type PpR24 to assess whether beneficial traits had been improved. Examinations into bacterial growth, colonisation of the phylloplane, and isolate motility were conducted.

Bacterial motility has been associated with biofilm surface attachment and bacterial virulence [162]. In several bacterial pathogens, many belonging to the enterobacteriaceae, flagella-mediated motility has been seen to correlate with pathogen virulence [180]. Flagella can play an important role in the early colonisation stages of a host but after colony establishment many species down-regulate flagella production in favour of systems supportive of a sessile lifestyle, such as exopolysaccharide (EPS) production [172, 180]. The association between virulence and motility is particularly well studied in the pathogen *Pseudomonas aeruginosa*, where the bacterium's use of flagellum and type IV pili are key to the colonisation and sustained presence in a host. A decrease in *P. aeruginosa* biofilm formation saw an increase in swarming behaviour [181, 182, 183, 184, 185]. Pathogens can also require maintained motility to thrive in a host. For example, *Helicobacter pylori* is a pathogen that inhabits the human gastric tract and relies on constant motility to remain infectious [180].

The wild-type PpR24 possesses a polar flagellum that may contribute to its aphicidal properties. Bacteria can use their flagellum for two main forms of

motility. Swimming motility can be defined as individual cells moving through a medium powered by rotating flagella [186], whereas swarming motility describes the flagella-driven, synchronised group movement of bacteria [187, 188]. Due to the inverse relationship of biofilm formation and motility, it is plausible that biofilm formers are affected in swimming and swarming motility, which in turn may affect aphid virulence [178].

3.2 Materials and Methods

The wild-type PpR24 was isolated in a previous study [8] and used as the ancestral strain to seed two evolutionary experiments. One experiment investigated whether biofilm formation could arise in a population (PpR24b isolates), the other whether improved aphid virulence would evolve (PpR24a isolates). In both experimental systems, ten independent lineages derived from the same wild-type PpR24 strain were serially passaged and final isolates randomly selected from plates spread with final passage bacteria.

3.2.1 Biofilm passage protocol

A biofilm passage lasted one week following the protocol as devised by Spiers *et al.*[175] (Figure 3.1), and were serially passaged for ten selection cycles.

The wild-type *Pseudomonas poae* PpR24 was used to seed the biofilm passages as the 'ancestor' isolate. PpR24 colonies were grown on a KA and Nitrofurantoin plate and a single colony picked and grown overnight. Ten glass universals of 10ml KB media were inoculated with the wild-type PpR24 from the overnight ancestral population. Five lineages of these universals, PpR24b1-b5 were inoculated with 10μl of bacteria and the other five, PpR24b6-b10 100μl of bacteria. Different volumes were used to explore whether a larger volume allowed more opportunity for adaptations to emerge. The microcosms were incubated with loose lids at 27°C for one week and were not shaken to allow biofilms to form at the air-liquid interface in aerobic conditions.

At the conclusion of each passage, microcosms were carefully removed from the incubator to ensure no biofilms were dislodged. Visual observations on the presence of biofilms were made. The passages were then continued in fresh KB media. 10μl of bacteria-broth suspension was removed from the air-liquid layer of the old microcosm and added to the new, fresh media. This was repeated for the 100μl samples. Frozen stocks were taken from each isolate, each passage to create a 'fossil record' of the bacteria's evolution. Once the ten passages for each of the derived ten isolates were complete, PCRs were conducted to ensure *P. poae* was recovered.

3.2.1.1 Bead test of biofilm strength

To test the strength, or maximum deformation mass (MDM) of the biofilms, 2mm glass beads weighing 0.012g were dropped onto the centre of the biofilm from a constant height until the biofilm sagged or broke. The more beads supported was taken to indicate a stronger biofilm.

3.2.1.2 Biofilm cellular attachment strength

Biofilm attachment strength was assessed using the crystal violet staining technique as laid out by OToole *et al.* [185]. After recording the biofilms' strength using the bead test described above, the glass universals containing the bacterial-broth suspension were emptied and stained with 1ml of 0.05% (w\v) Crystal Violet (Figure 3.1). The universals were then agitated for one minute and gently rinsed out with water. The stain was then eluted with 5ml ethanol, shaken for 15 minutes and the optical density recoded at OD600. The higher the optical density measured indicated a higher cellular attachment to the universal wall.

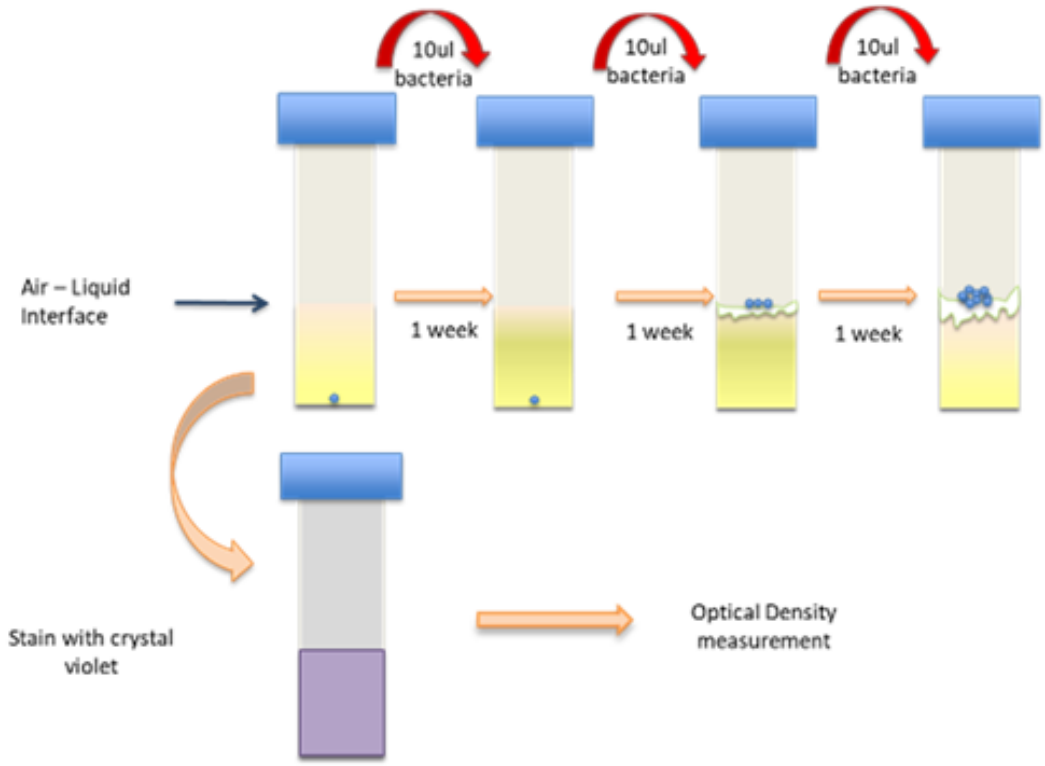


Figure 3.1: Biofilm passage protocol to assess maximum deformation mass (MDM) and bacterial attachment strength. Isolates were left to grow in KB broth for one week at 27°C without agitation. After a week had elapsed, the maximum deformation mass of potential biofilms formed at the air-liquid interface was tested by dropping 2mm diameter glass beads in to the centre of the biofilm from a consistent height. The mass supported indicated the strength of the biofilm. To assess bacterial attachment strength, passage microcosms were emptied after MDM measurements were taken and stained with 1ml of 0.05% (w\v) Crystal Violet. The universals were agitated for one minute and gently rinsed out with water. The stain was then eluted with 5ml ethanol, shaken for 15 minutes and the OD600 recorded.

3.2.2 Aphid passage protocol

Ten aphid mortality sachets were prepared (as in Chapter 2.5) using the ancestral wild-type *P. poae* PpR24 as the inoculum in each sachet. After 48 hours, the aphids were recovered from each sachet and surfaced sterilised with 1% sodium hypochlorite solution for 5 minutes in 1.5ml eppendorfs. The aphids were then rinsed 3 times with sterile water, centrifuging washes at 5000rpm for 3 minutes. After removing the sterile water, 200μl of sterilised PBS was added

to the eppendorfs and the aphids were homogenised using sterile micropestles.

Each of the ten samples were aliquoted in 10μl droplets and spread onto KB and Nitrofurantoin plates with glass spreaders. The plates were incubated at 27°C and left to grow lawns overnight. After incubation, 1ml of PBS was pippetted onto the plate to loosen the lawns and to enable the bacteria to be collected for the next passage. Frozen stocks of each derived isolate were made from this suspension and the next passage sachets made from these stocks. Ten passages were conducted for each of the derived ten isolates and PCRs to ensure *P. poae* was recovered were carried out (Figure 3.2).

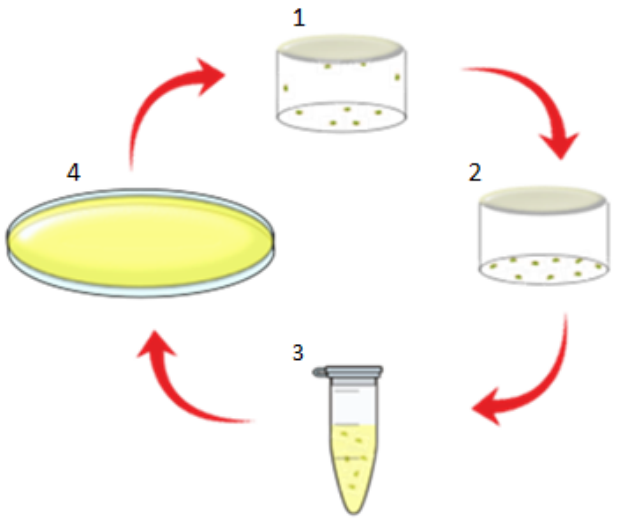


Figure 3.2: Aphid passage protocol. 1) Aphid feeding sachets were inoculated with bacteria at a cellular suspension of 10^7 CFU mL^{-1}. Ten aphids were added to each sachet. 2) After being left to feed for 48 hours, dead aphids were recovered and surfaced sterilised. 3) The recovered aphids were homogenised with micropestles in sterilised PBS. 4) The aphid-bacterial slurry was plated onto a KA and Nitrofurantoin plate and incubated for 16 hours. The bacteria grown were used to inoculate the next passage.

3.2.3 Examination of trade-offs

After undergoing 10 passages for either biofilm formation or aphid-killing, an assessment of whether trade-offs had occurred between traits was carried out with the final evolved isolates. Each isolate was tested in each of the following experimental procedures three times in triplicate.

3.2.3.1 Aphicidal properties and biofilm formation

Final strain biofilm-passaged bacteria were tested for virulence against aphids following the same sachet methodology as in Chapter 2, Section 2.5. Likewise, aphid-passaged final isolates were tested for biofilm formation as in Section 3.2.1. Comparisons were made between all evolved isolates and the wild-type PpR24.

3.2.3.2 Bacterial growth

Final passage isolates were tested for changes in growth from the wild-type PpR24 when in a broth environment. Isolates from single colonies were grown overnight and resuspended in PBS at an OD to 1. In a 96 well plate, 180μl of KB broth was added to each well and 20μl of culture. Growth readings were taken using a Bioscreen C plate reader, at 27°C using a 600nm filter. Readings were taken every 20 minutes over 24 hours in continuously shaken and a static environment.

3.2.3.3 Motility assay

Whether the derived isolates differed in motility compared to the wild-type PpR24 was investigated. Two methods of bacterial motility, swimming and swarming, were examined. Isolates were streaked to single colonies on KB agar plates and grown overnight at 27°C. For swimming, each petri dish contained 30ml of semi-solid, 0.25% w/v agar with 10% KB. For swarming plates, 0.25% w/v agar with full strength KB was used. Plates were inoculated with strains by stabbing the centre of the plate, taking care not to penetrate through the agar to the bottom. Plates were imaged at 0, 12, 24, 36 and 48 hours and incubated at 27°C between imaging. Images were taken using a G box and analysed using ImageJ software [189].

3.2.3.4 Bacterial colonization on plant assay

Bacterial spray suspensions of the wild-type and passaged isolates were made following the protocol in Chapter 2, Section 2.6, and applied at a cellular suspension of 10^7 CFU mL^{-1}.

Samples were taken on days 0, 1, 3, 7, 14, and 21. Leaf disks, $1cm^2$, were excised from the plants and placed in sterile Eppendorf tubes containing 200μl

sterile PBS. Leaf disks were macerated using micropestles and vortexed for five seconds. Dilution series were made, pipetting 10μl of each dilution onto KA and nitrofurantoin plates in triplicate. Plates were left to grow for 16 hours at 27°C and colony counts made and averaged to determine CFU per leaf area.

3.2.4 Statistical analysis

Statistical tests were conducted in R [190], version 3.6.1. and GraphPad Prism 8 [191], version 8.4.1. Kruskal-Wallis multiple comparison with a post-hoc Dunn test was used to analyse statistical differences between derived isolates. p-values were adjusted with the Bonferroni method to account for multiple comparisons.

3.3 Results

3.3.1 Experimental evolution passages

3.3.1.1 Biofilm passage assay

Biofilm formation can improve bacterial colonisation and survival on plant surfaces [164, 165]. As such, biofilms may prove a valuable trait to improve performance of bacterial biocontrol agents applied as foliar sprays, as well as contribute to occlusion in the gut of target insects [9, 122]. On this hypothesis, an experimental evolution approach was applied to evolve biofilm-forming phenotype of PpR24. The wild-type PpR24 does not form biofilms in a broth suspension, therefore experimental serial passages were used in a bid to evolve a biofilm formation. Following the methodology described in Section 3.2.1., PpR24 isolates were serially passaged in static KB broth for the duration of ten cycles. For each passage, cellular attachment to glass universals and biofilm strength were recorded for each microcosm as an indicator of biofilm presence before frozen stocks were made. After ten passage cycles, statistical assessment was carried out on the ten final-passage evolved isolates and comparisons in isolate performance were made.

Over the course of the experimental passages, out of the ten lineages only five intermittently produced biofilms capable of supporting any mass in the form of pellicles at the air-liquid interface: isolates PpR24b1, PpR24b2, PpR24b4, PpR24b5, and PpR24b6. However, at the conclusion of the final passage, mass-supporting biofilms were only present in the microcosms of PpR24b4, PpR24b5, and PpR24b6. Final-passage isolates PpR24b4 and PpR24b5 exhibited a loss of pigmentation after one week of growth compared to the eight other biofilm lineages. The bacterial-broth of PpR24b4 and PpR24b5 appeared transparent with a yellow tint, with a thick pellicle at the air-liquid interface. In contrast, for all other final-passaged isolates the bacterial-broth resembled that of the wild-type PpR24; an opaque, yellow-green, with a thick meniscus (Figure 3.3).

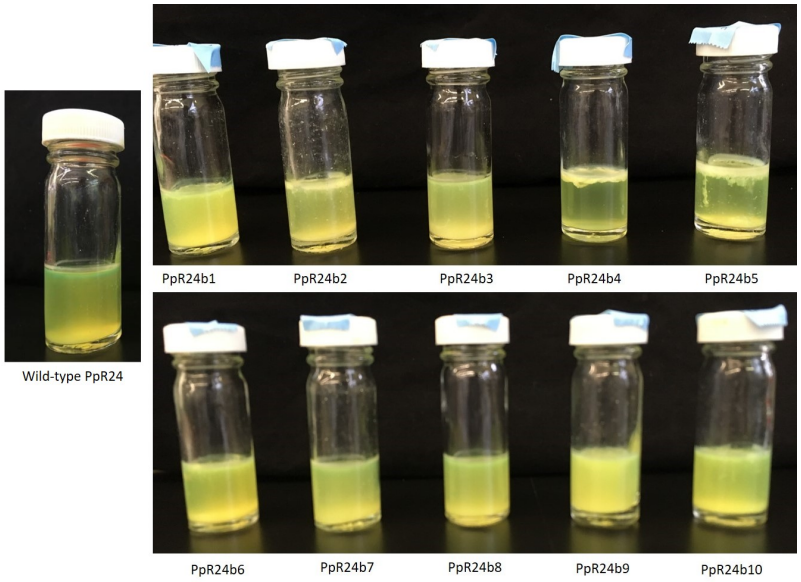


Figure 3.3: Biofilm-passaged isolate microcosms at the conclusion of ten passages. Sterile KB broth was innoculated with 10μl of bacteria from the previous passage in isolates PpR24b1-5, and 100μl in PpR24b6-10. Isolates were grown for a week at 27°C with no agitation and loose lids. A loss of pigmentation was observed in isolates PpR24b4 and PpR24b5 whereas the other microcosms retained their opaque-yellow pigment seen in the wild-type PpR24.

The biofilm pellicles of isolates PpR24b4 and PpR24b5 differed in structure. PpR24b4's biofilm had a dense, folded appearance whereas isolate PpR24b5's biofilm was thin and flaky, with a foamy meniscus and precipitate gathered at the bottom of the microcosm, most likely from unsupported biofilm matter (Figure 3.4).

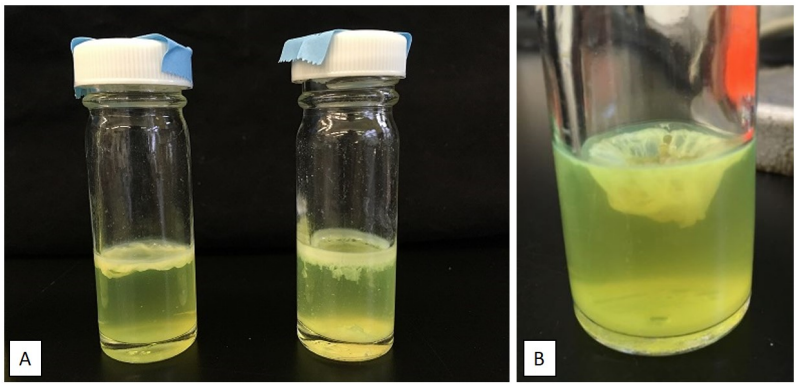
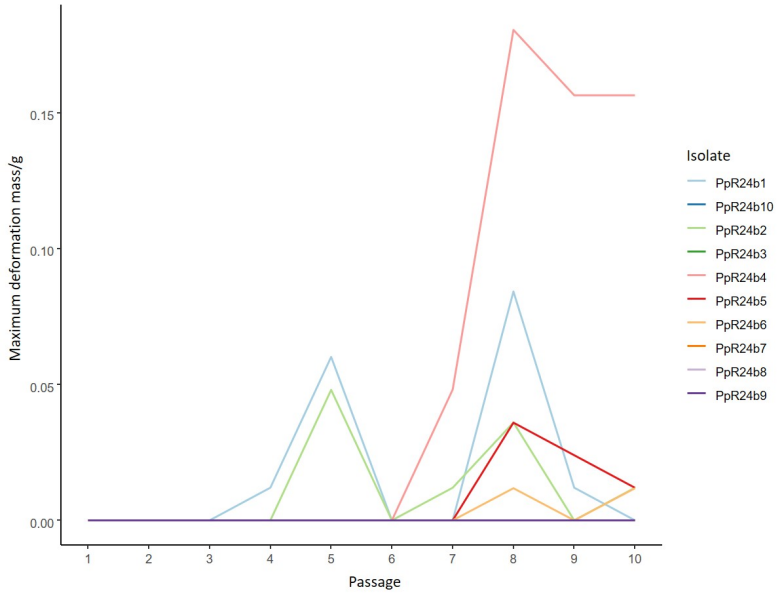


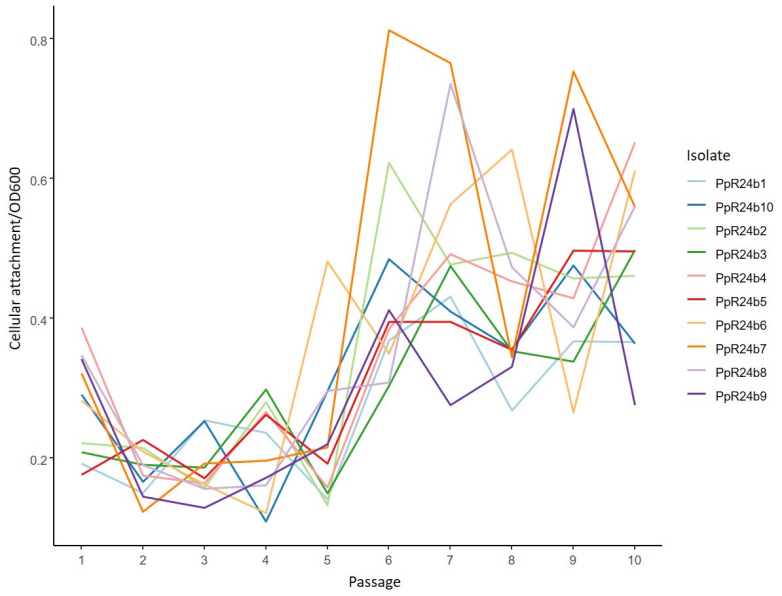
Figure 3.4: Microcosms of isolates PpR24b4 and PpR24b5 at the conclusion of ten passages. (a) Isolate PpR24b4 (left) formed the strongest biofilm and had a folded, transparent appearance as a pellicle at the air-liquid interface. PpR24b5 (right) was unable to support as much mass as isolate PpR24b4. The biofilm produced had a grainy appearance with a foamy meniscus and a large amount of precipitate a the bottom of the microcosm. (b) Example of testing the maximum deformation mass of isolate PpR24b4.

Isolates PpR24b1, PpR24b2, PpR24b5, and PpR24b6 fluctuated in their biofilm strength between passages. Isolates PpR24b1 and PpR24b2 first formed biofilms in passage 4 and 5 respectively but both failed to form a biofilm of capable of supporting beads in passage 6. However, biofilms subsequently formed again in passages 7 and 8, as was the case for isolates PpR24b6 and PpR24b5. In contrast, biofilm formation emerged at passage 7 in isolate PpR24b4, which consistently formed strong biofilms capable of supporting multiple beads until the conclusion of the experiment (Figure 3.5a).

Cellular attachment to the universal walls (Figure 3.5b) showed a positive trend across all biofilm lineages, regardless of biofilm strength and pellicle formation. This may suggest that improved bacterial adhesion may be an adaptation of survival in a broth environment.



(a) Biofilm maximum deformation mass



(b) Biofilm attachment strength

Figure 3.5: Biofilm evolution over ten passages. (a) Maximum deformation mass of biofilm passaged isolates. Biofilm strength fluctuated over the course of the ten passages, with biofilms capable of supporting any mass arising in only five of the ten isolate lineages. At the conclusion of the serial passages, isolate PpR24b4 was the strongest biofilm with the highest maximum deformation mass. (b) Biofilm attachment strength. Cellular attachment of evolved isolates showed generally increased over the course of the ten passages for all isolates, regardless of biofilm strength. For each time-point, there is one given data point per isolate lineage.

3.3.1.2 Bacterial virulence assay

On the hypothesis that improving bacterial fitness inside the aphid may enhance bacterial virulence by increasing bacterial growth and, consequently, toxin production, ten lineages of PpR24 isolates were serially passaged through aphids via inoculated diet, recovered from the macerated aphid and grown and used to seed the subsequent passage (as described in Section 3.2.2.).

Over the course of ten passages, all ten lineages exhibited stochastic fluctuations in aphid mortality at 48 hours. No discernible trends in each isolate lineage were observed (Figure 3.6), therefore it may be inferred that no improved virulence traits evolved and established in the isolate populations.

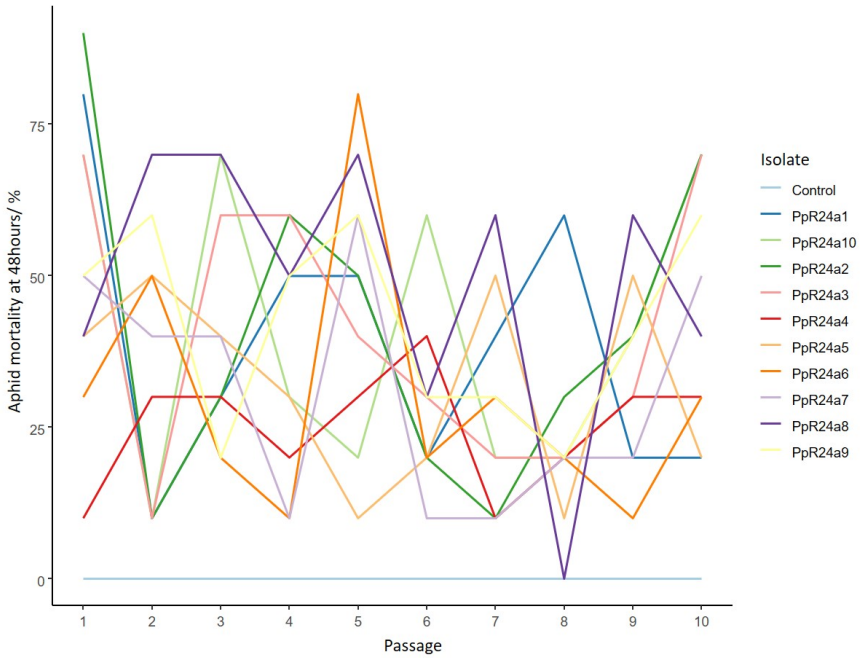


Figure 3.6: Percentage aphid mortality at 48 hours for each of the 10 passages. Aphid Mittler diet was inoculated with bacteria at a cellular suspension of 10^{-7} CFU mL^{-1}. Ten aphids were left to feed on a sachet for 48 hours, with aphid mortality recordings taken at 0, 1, 6, 18, 24, 30, 42, 46 and 48 hours. Dead aphids were recovered after 48 hours, surface sterilised, homogenised in PBS and the bacterial-aphid slurry plated onto KA and nitrofurantoin plates. After 16 hours of growth, bacteria was recovered from the plate and used to inoculate the next passage. No discernible trend in aphid mortality was observed in any isolate lineage. For each time-point, there is one given data point per isolate lineage.

3.3.1.3 Significant changes within evolution passages

To assess whether the two evolution passages were successful in evolving different phenotypes, z scores were used to test for statistical differences between final isolates within each lineage. Results for biofilm attachment, biofilm strength and aphid virulence were standardised to z scores using the equation:

$$z = \frac{x - \mu}{\sigma} \tag{3.1}$$

where μ is the mean and σ the standard deviation. Scores were considered statistically different from the rest if they were outside two standard deviations from the mean.

Isolate PpR24b4 was the only isolate to exhibit a statistically different phenotype, having a statistically stronger maximum deformation mass than the other evolved isolates passaged for biofilm formation. No statistical differences in biofilm cellular attachment strength were observed between the final evolved biofilm-passaged isolates and no statistical differences in aphid virulence were seen for final aphid-passaged isolates (Figure 3.7).

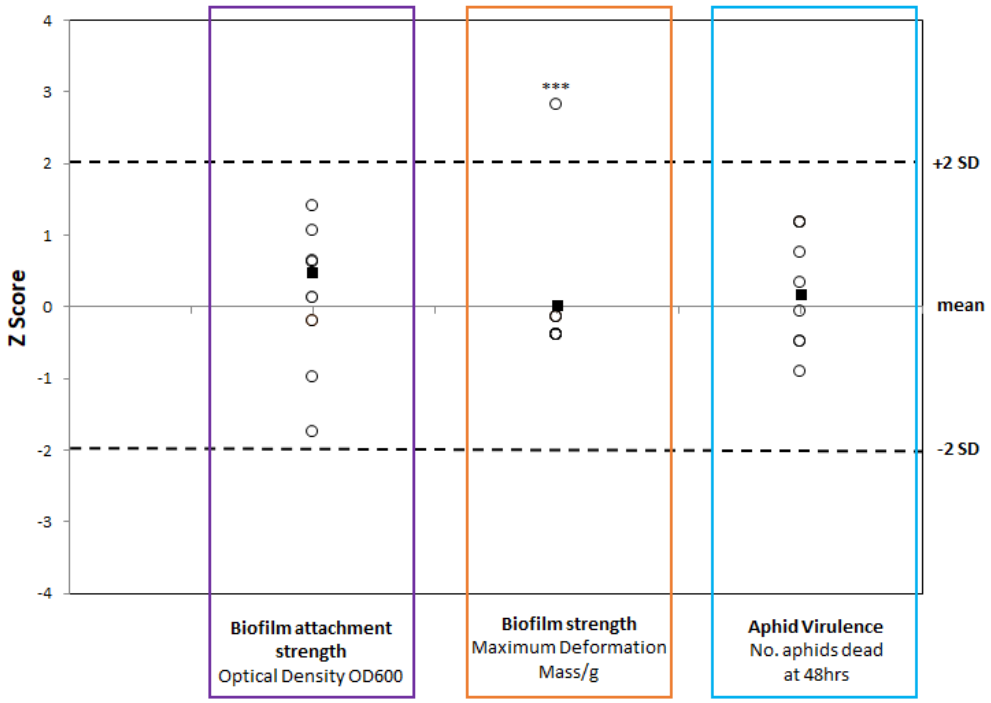


Figure 3.7: Passage results for the final isolates of the three traits we hoped to evolve after 10 passages. Results were standardised as z scores to allow significant differences between isolates to be observed. Black squares are the mean of each group and circles individual isolates. Only one isolate, PpR24b4, exhibited a statistically different phenotype to the other biofilm-passaged isolates when tested for biofilm strength as it was more than two standard deviations from the mean.

3.3.2 Analysis of trade-offs between isolates

At the conclusion of the serial passages for the two evolutionary lineages, comparisons between the final isolates of the biofilm and aphid serial passages and the wild-type PpR24 were carried out to assess whether any improvements to isolate performance had evolved that may be of benefit to a potential biocontrol agent. Although no significant difference in aphid virulence was observed between the final aphid-passage isolates, it is possible that changes occurred in other traits associated with virulence and bacterial survival. Furthermore, biofilm formation and adaptations to the broth-environment may have resulted in trade-offs with traits important in bacterial establishment in a crop, such as motility and growth rate, and may have affected aphicidal properties.

3.3.2.1 Trade-off in aphid virulence

The wild-type PpR24 killed on average 70% of aphids in 48 hours [9]. The primary aim of the experimental passages was to evolve isolates with improved aphid-virulent properties therefore using the same inoculated artificial diet sachet system as described above (Section 2.5 and Subsection 3.2.2), comparisons in aphid mortality between the wild-type PpR24 and all the final-passage derived isolates from the aphid and biofilm passages were made. A significant statistical difference in aphid mortality was observed between the different isolate treatments (Kruskal-Wallis test p-value <0.0001).

Lower mortality levels were observed in aphids fed diet treated with biofilm-passaged isolates when compared to the wild-type and aphid-passaged isolates (Figure 3.8), which may suggest a trade-off occurred between virulent properties and survival in the broth environment of the passages (Figure 3.8). However, Dunn's post-hoc analysis found only two isolates, PpR24b1 and PpR24b2, to be statistically different to the wild-type (adjusted p-values <0.05). Both PpR24b1 and PpR24b2 intermittently produced strong biofilms over the course of the serial passages therefore it is possible there is a trade-off between biofilm formation and aphid-virulence. Isolate PpR24b4, which evolved the strongest biofilm out of the passaged isolates, was not found to cause a statistically lower aphid mortality in post-hoc analysis when compared to the wild-type (adjusted p-value = 0.2044), although by eye the isolate conferred poorer aphid-killing when compared to the wild-type PpR24. However, PpR24b4 had a statistically lower aphid-mortality than isolates PpR24a1 (adjusted p-value = 0.0318) and PpR24a2 (adjusted p-value = 0.0282). For complete post-hoc pair-wise differences, see Appendix B, Table B.1.1.

The wild-type and all aphid-passaged isolates, aside from PpR24a8, had a statistically higher aphid-mortality than the PBS control sachets (adjusted p-values <0.05). However, post-hoc analysis found no statistical differences between the wild-type and ten aphid-passaged isolates (adjusted p-values >0.999), from which it could be inferred no significant improvement to aphid-virulence occurred over the course of the experimental passages through aphids.

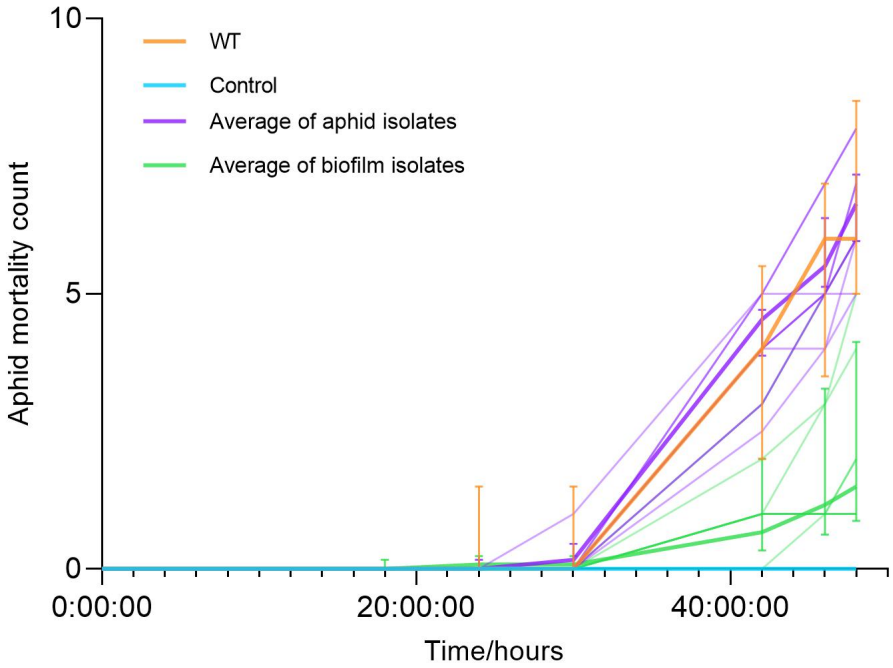


Figure 3.8: Trade-off in aphid virulence. Aphid mortality was tested for the wild-type PpR24, PBS control and all the final derived isolates from the biofilm and aphid passages. Mittler diet was inoculated with bacteria at a cellular suspension of 10^{-7} CFU mL^{-1}. Ten aphids were left to feed on a sachet for 48 hours, with aphid mortality recordings taken at 0, 1, 6, 18, 24, 30, 42, 46 and 48 hours (n=9). All biofilm-passaged isolates caused a lower aphid-mortality than the wild-type and aphid-passaged isolates, whereas no statistically significant difference was found between the wild-type and aphid passaged isolates. The median for each isolate is presented here, with bars for the interquartile range only present on the average for isolates and wild-type for aesthetic reasons (see Appendix B for complete interquartile range bars).

3.3.2.2 Trade-off in biofilm strength and attachment strength

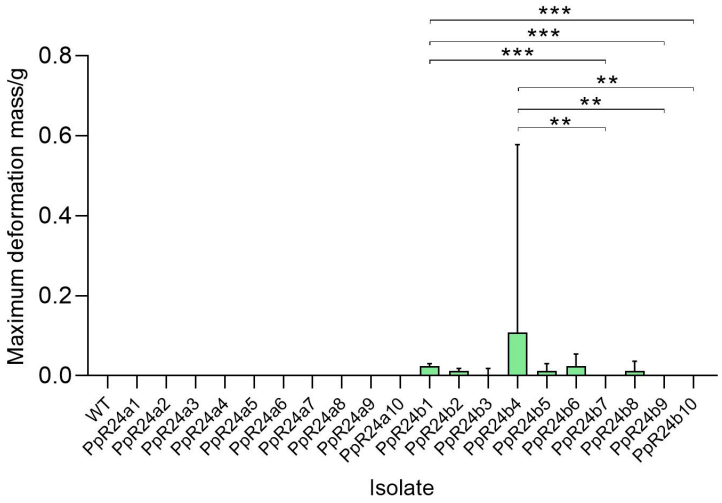
As discussed in Section 3.3.1, an experimental evolution approach serially passaging PpR24 isolates in broth proved successful in evolving a biofilm phenotype, particularly in isolate PpR24b4. Using the same methodology as the serial passages (see Section 3.2.1), all final-passage isolates from biofilm and through-aphid passages, and the wild-type PpR24, were left to grow in KB broth for a week and comparisons on biofilm strength and attachment strength were made. The wild-type PpR24 and all aphid-passaged isolates were unable to form biofilms capable of supporting any mass, whereas the biofilm-passaged

isolates generally had a higher maximum deformation mass and attachment strength (Figure 3.9).

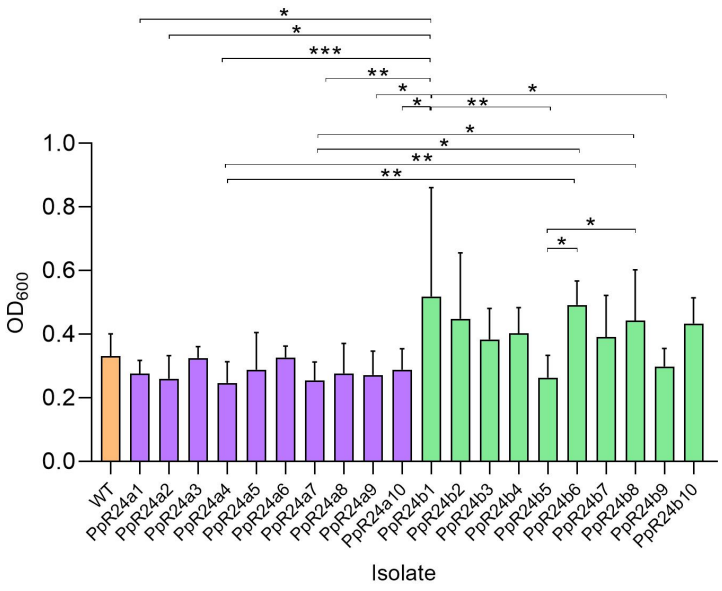
A significant statistical difference in maximum deformation mass between isolates was observed (Kruskal-Wallis test p-value <0.0001). Dunn's post-hoc analysis found no significant differences in the biofilm strength of the wild-type and aphid-passaged isolates as neither were able to support any mass. However, only two biofilm-passaged isolates, PpR24b1 and PpR24b4, were found to be statistically different to the wild-type and all aphid-passaged isolates (adjusted p-values <0.05). PpR24b1 intermittently produced strong biofilms over the course of the serial passages and PpR24b4 evolved the strongest biofilm of all the biofilm-passaged isolates. PpR24b1 and PpR24b4 also formed statistically stronger biofilms than isolates PpR24b7, PpR24b9 and PpR24b10, which did not form biofilms over the course of the evolutionary passages therefore it may be inferred that no changes from the wild-type occurred in these isolates (Figure 3.8). For complete post-hoc pair-wise differences, see Appendix B, Table B.2.

Unsurprisingly, biofilm attachment strength was generally higher for biofilm-passaged isolates compared to the aphid-passaged and wild-type isolates. However, isolates PpR24b5 and PpR24b9 had noticeably reduced attachment compared to the other biofilm isolates. A significant statistical difference between isolate attachment was observed (Kruskal-Wallis test p-value <0.0001) and significant pairwise differences can be seen in Figure 3.9 (for complete post-hoc pair-wise differences, see Appendix B, Table B.3). Interestingly, although PpR24b4 had the strongest maximum deformation mass, it did not have the highest cellular attachment. Isolates PpR24b1, PpR24b2, PpR24b6, PpR24b8, and PpR24b10 all had higher levels of attachment.

With regards to differences in biofilm formation between biofilm-passaged isolates serially passaged with 10μl or 100μl of bacterial-broth, no consistent significant differences were observed in biofilm formation between the two volumes. Post-hoc analysis found isolates PpR24b1 and PpR24b4, both 10μl passaged isolates, had a statistically higher maximum deformation mass than isolates PpR24b7, PpR24b9 and PpR24b10, all three of which were 100μl passaged isolates and unable to produce mass supporting biofilms. However, although not statistically significant, 100μl passaged isolates PpR24b6 and PpR24b9 did form mass-supporting biofilms therefore it could be inferred that biofilm formation was not dependent on inoculation volume.



(a) Biofilm maximum deformation mass



(b) Biofilm attachment strength

Figure 3.9: Biofilm trade-off analyses. (a) Trade-off biofilm strength. Maximum deformation mass of all final biofilm-passaged and aphid-passaged isolates compared to the wild-type PpR24. The mass supported indicated the strength of the biofilm. The median and interquartile range of isolates are presented, with significant pairwise differences between biofilm isolates present (n=9). Isolates PpR24b1 and PpR24b4 were statistically different to the wild-type and all aphid-passaged isolates but for aesthetic reasons comparison bars were excluded. (b) Trade-off biofilm attachment strength. Comparisons of cellular attachment to glass universals of all final biofilm-passaged and aphid-passaged isolates compared to the wild-type PpR24. The median and interquartile range of isolates are presented, with significant pairwise differences (n=9). (Full pair-wise differences found in Appendix B, Section B.1.2.)

3.3.2.3 Colony morphology

Biofilm formation has been associated with a change in colony appearance, such as in the case of the SBW25 WS phenotype that formed distinctive wrinkled-colonies on agar plates in contrast to the smooth, round appearance of the wild-type [178]. In the current study, colony morphology of the final evolved isolates from the aphid and biofilm passages were examined but little variation was found between the wild-type PpR24 and evolved isolates. However, the strong biofilm-forming isolate, PpR24b4, had reduced plate growth when compared to the wild-type as well as smaller, more concise circular colonies than the wild-type (Figure 3.10).

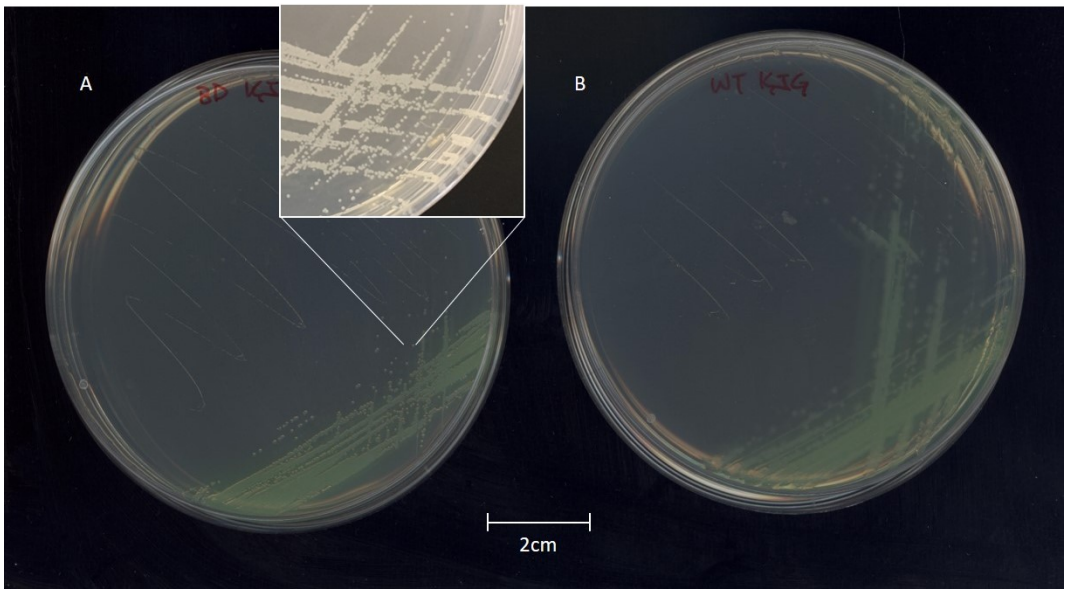


Figure 3.10: A comparison in colony morphology between A) biofilm forming isolate PpR24b4 and B) wild-type PpR24 after 16 hours when grown on KA plates. Isolate PpR24b4 formed smaller, more concise colonies than the wild-type and had a reduced spread of growth.

3.3.2.4 Growth assay

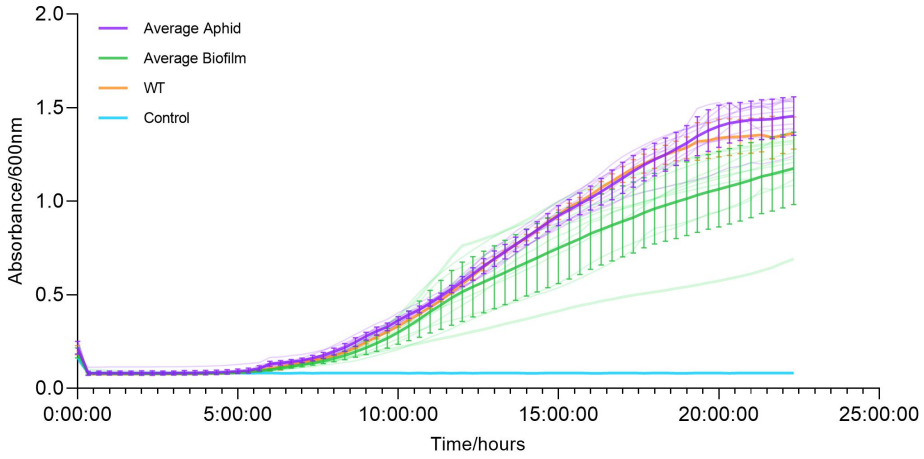
An investigation into whether changes in bacterial growth rate had occurred in the evolved isolates was assessed as this may have knock-on effects on the isolates' virulence and plant colonisation.

When left to grow in static conditions, at 24 hours the majority of biofilm-passaged isolates had lower levels of absorbance when compared to the aphid-passaged and wild-type isolates, which was taken to indicate a lower density of

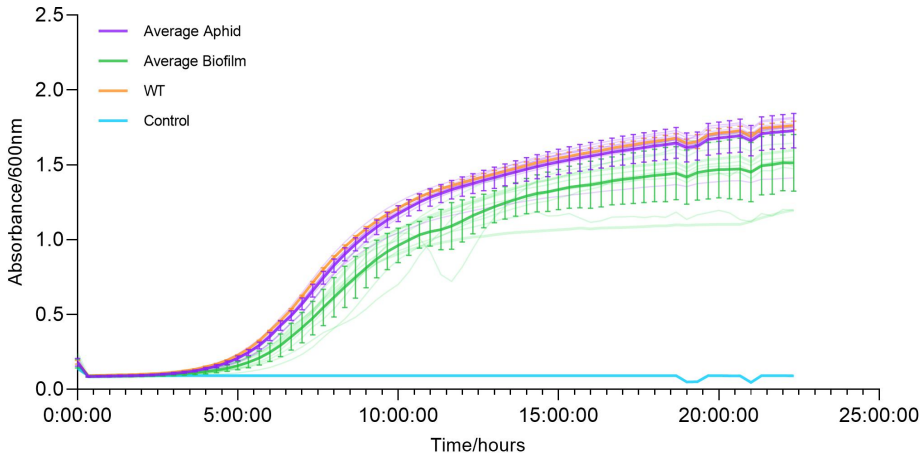
bacterial cells. A statistical difference was present in isolate growth (Kruskal-Wallis test p-value <0.0001), however Dunn's test post-hoc analysis found no significant differences between absorbance levels in the final strain isolates, suggesting no significant differences in isolate growth. When the results are examined by eye, all biofilm-passaged isolates reached lower cellular levels than the wild-type and aphid-passaged isolates. Biofilm-forming isolate PpR24b9 in particular had reduced growth when compared to other tested isolates (Figure 3.11a).

Similar results were seen for isolate growth in a shaken environment. Aside from aphid-passaged isolate PpR24a10, all biofilm isolates achieved lower levels of growth at 24 hours than the wild-type and aphid-passaged isolates. A significant difference was found between isolates (Kruskal-Wallis test p-value <0.0001) at 24 hours but post-hoc comparisons found no significant differences between individual isolates. That being said, it appears the biofilm isolates PpR24b4 and PpR24b9 reached the stationary phase at a lower population than the other isolates (Figure 3.11b).

As isolate PpR24b4 formed thick, strong bioiflms in static conditions, it is possible that PpR24b4 is unable to colonise broth in shaken conditions as effectively as the other isolates. Alternatively, due to its strong biofilm properties potentially as an adaptation to the broth environment, PpR24b4 may exhaust the nutrients in the broth faster than the other, non-biofilm forming isolates in order to produce the various adhesives and exopolysaccharides required for biofilm formation and thus achieve lower cell densities. However, it is unclear as to why such noticeably lower levels of absorbance are absent in the static broth environment for this isolate. With regards to isolate PpR24b9, it is possible that the poorer performance in cellular attachment and biofilm strength when compared to other biofilm-passaged isolates can be attributed to its reduced growth.



(a) Static



(b) Shaken

Figure 3.11: Evolved isolate and wild-type PpR24 growth in (a)static conditions, and (b) continuously shaken conditions (n=4). For both static and shaken environments, biofilm-passaged isolates had reduced growth compared to the aphid-passaged and wild-type PpR24 isolates. Average growth for aphid-passaged and biofilm-passaged isolates with interquartile range bars are presented (Graphical representation of interquartile range bars for all isolates can be found in Appendix B).

3.3.2.5 Motility assay

Bacterial motility can be an important factor in virulence and successful establishment in a new environment [180], therefore an assessment to whether any changes in evolved isolate swimming or swarming motility had occurred was carried out. Measuring the area of bacterial spread of isolates on swimming

and swarming agar resulted in biofilm-passaged isolates having a consistently lower area of spread when compared to the aphid-passaged and wild-type isolates (an example of isolate spread can be found in Figure 3.12).

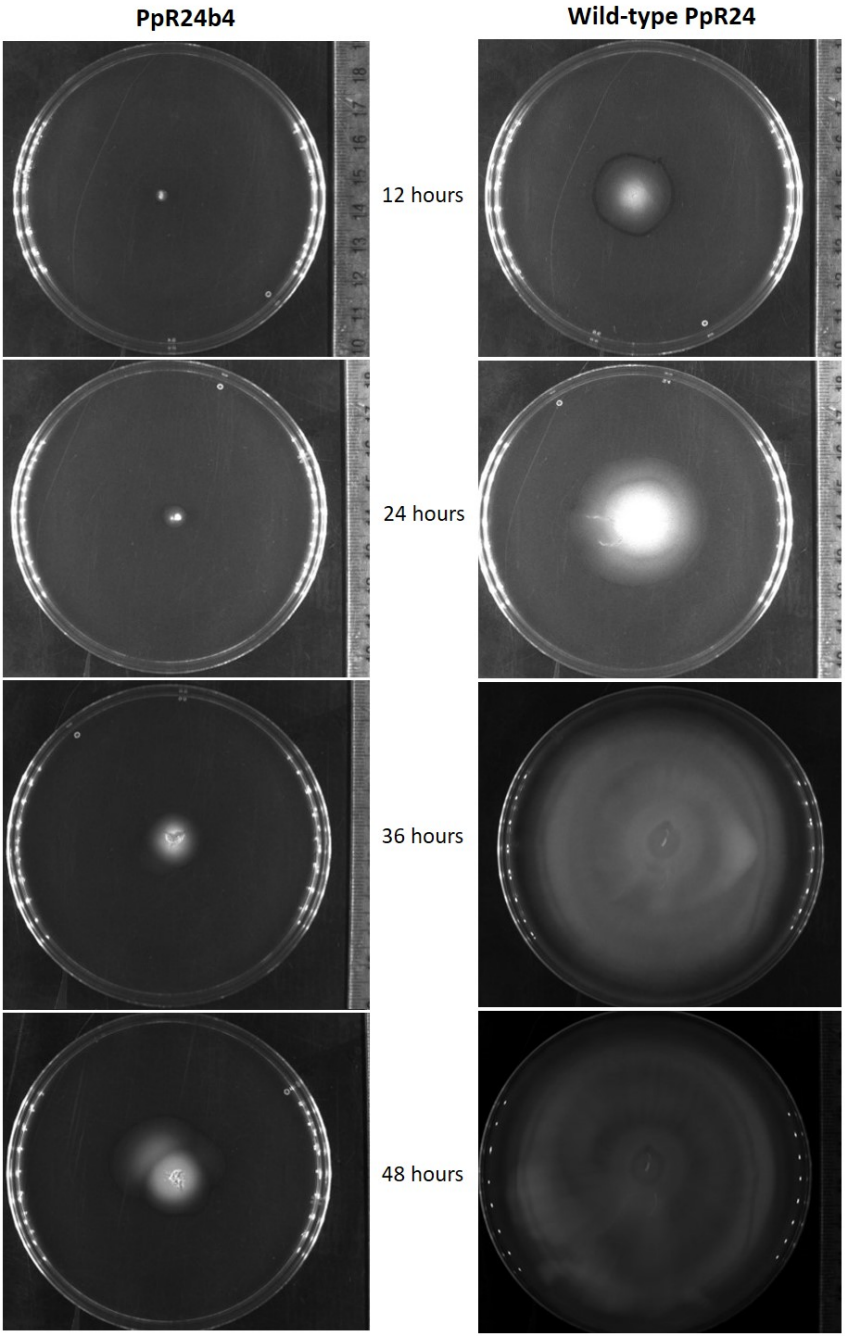
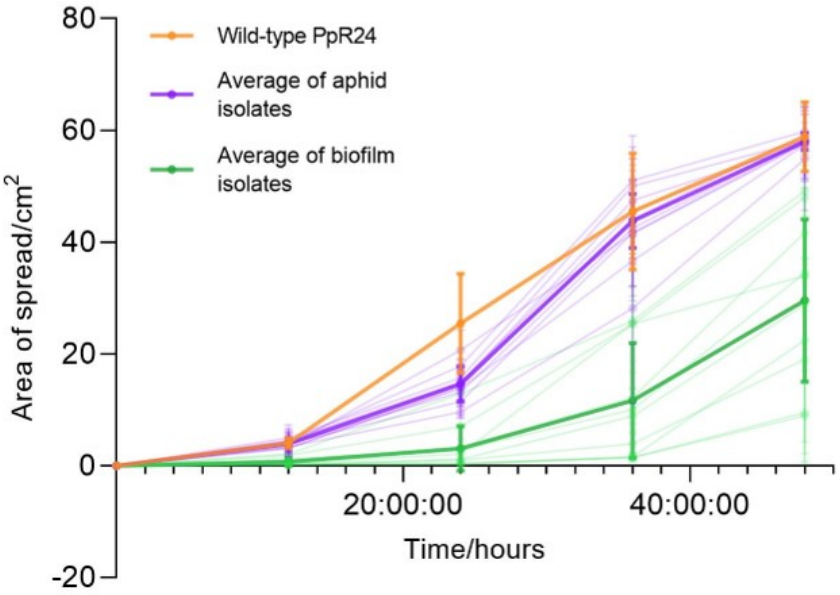


Figure 3.12: Swarming motility of isolate PpR24b4 and the wild-type PpR24. Over 48 hours, PpR24 showed reduced growth compared to the wild-type PpR24.

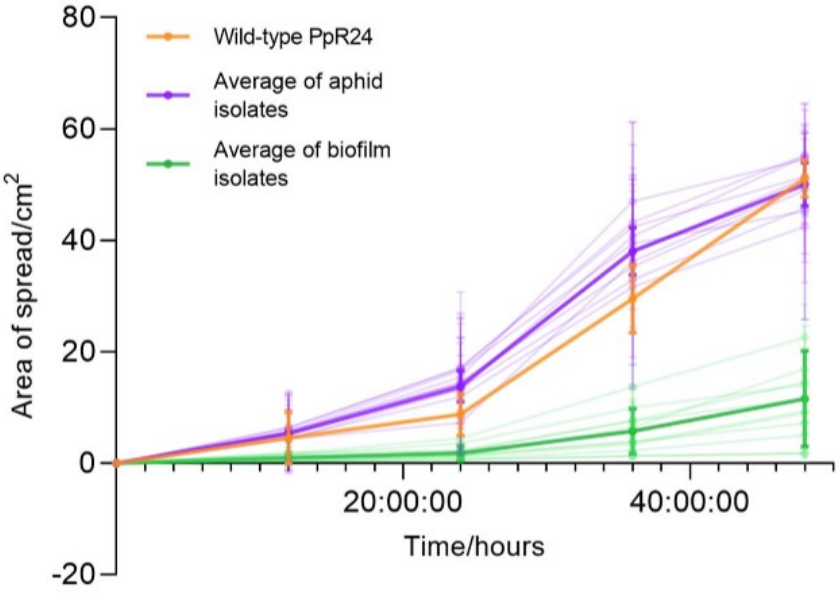
With regards to swimming motility (Figure 3.13a), Kruskal-Wallis analysis found a statistical difference between isolate area of spread (Kruskal-Wallis test p-value <0.0001). Post-hoc analysis found no significant differences between the wild-type and aphid-passaged isolates but the wild-type had significantly larger areas of spread when compared to biofilm-passaged isolates PpR24b2, PpR24b6, PpR24b8, and PpR24b10 (adjusted p-values <0.05). Isolates PpR24b2 and PpR24b6 also had statistically smaller areas of spread when compared to all aphid-passaged isolates.

When inoculated on swarming agar, aphid and biofilm-passaged isolates show a stark difference in motility, with bioiflm-passaged isolates all having reduced spread compared to aphid-isolate spread and the wild-type (Figure 3.13b). Kruskal-Wallis analysis found a statistically significant difference between isolate spread (Kruskal-Wallis test p-value <0.0001), which was further elucidated with Dunn's post-hoc analysis. Similarly to the swimming assay, isolates PpR24b2, PpR24b6, and PpR24b8 were found to have significantly less spread on the agar plates than most aphid isolates (for full post-hoc pair-wise differences, see Appenix B, Section B.1.3).

However, the biofilm isolates found to be statistically less motile in both swimming and swarming agar were not the strongest biofilm formers. As all biofilm isolates were less motile in both assays, regardless of strong biofilm formation, it could be inferred that the loss of motility is associated with adaptations to the broth environment through which the isolates were passaged, rather than due to biofilm formation.



(a) Swimming motility



(b) Swarming motility

Figure 3.13: Isolate motility. PpR24 isolate spread in (a) 30ml semi-solid, 0.25% w/v agar with 10% KB swimming agar, and (b) 30ml semi-solid, 0.25% w/v agar with full-strength KB swarming agar (n=9). No statistically significant differences were observed in the area of bacterial spread between the wild-type PpR24 or aphid-passaged isolates for both swimming and swarming motility. However, biofilm isolates consistently did not spread as much as the evolved aphid-passaged isolates and wild-type PpR24 in either mode of motility. Average growth for aphid-passaged and biofilm-passaged isolates with interquartile range bars are presented (Graphical representation of interquartile range bars for all isolates can be found in Appendix B, Section B.1.3).

3.3.2.6 Bacterial persistence on sweet pepper plants

As the bacteria are intended for use in a crop environment, it was important to assess whether any trade-offs had occurred that may affect the bacterium's ability to colonise the target plant. When applied as a foliar spary, the wild-type PpR24 was able to persist on the plant phylloplane for 21 days [9]. Following the foliar spray methodology in Section 2.6, the strongest biofilm-forming isolate, PpR24b4, was assessed for persistence on *C. annuum* over the course of 21 days to investigate whether any improvements had been made upon the wild-type in terms of bacterial colonisation, on the assumption that a biofilm phenotype may aid bacterial survival on the phylloplane. An aphid-passaged isolate was also tested, although as no significant differences were found between aphid-passaged isolates in all aforementioned trade-off assays, isolate PpR24a1 was selected at random to test for plant survival.

After foliar spray application, the wild-type PpR24, isolate PpR24a1, and PpR24b4 were all present on the plant for the duration of the 21 day experiment (Figure 3.14). Colony counts of bacteria recovered from the the internal and external leaf surfaces fluctuated over the course of the assay. All three isolates saw a stark drop in population from day 0 to 1, but a general positive trend in bacterial presence on the leaf continued until day 21. However, at the conclusion of the experiment on day 21, Kruskal-Wallis with Dunn post-hoc analysis found no significant difference between the bacterial populations present on the phylloplane at day 21 (p-value >0.05).

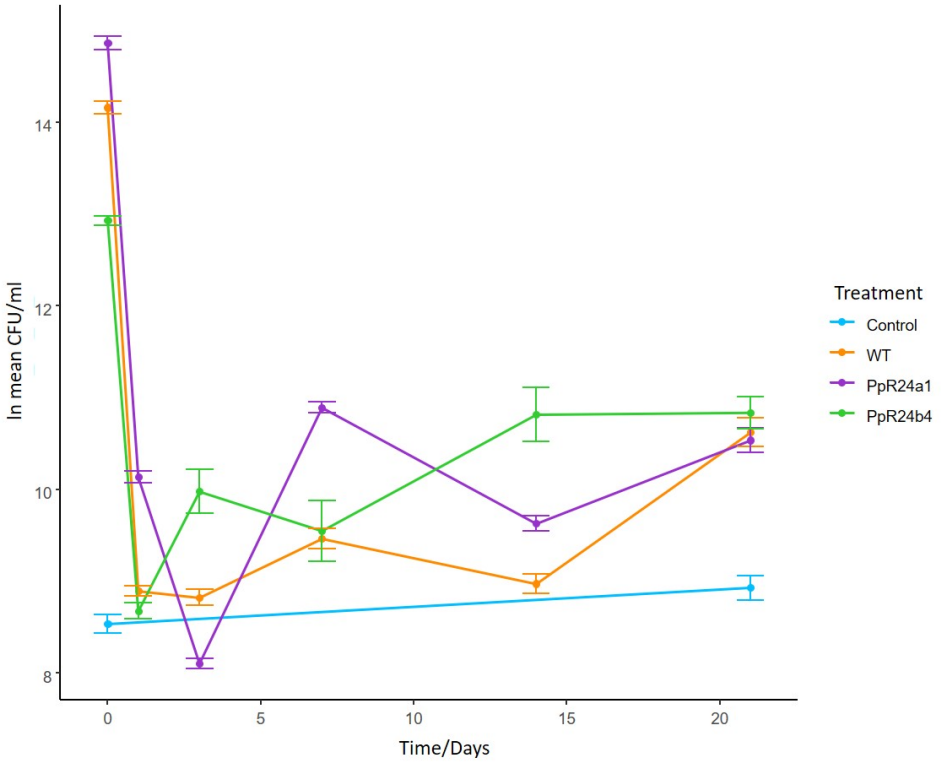


Figure 3.14: Bacterial isolate persistence on *Capsicuum annum*. Bacteria were applied via a foliar spray at a cellular suspension of 10^{-7} CFU mL^{-1}. At 0, 1, 3, 7, 14 and 21 days, leaf disc samples were taken in triplicate for each treatment and homongenised in PBS before plating on KB and nitrofurantoin plate for bacterial enumeration (n=9).

3.4 Discussion

Novel aphidcidal biopesticides are in high demand and experimental evolution may offer a means of evolving more efficient microbial-based aphid control without the stigma of genetically modified organisms. In this chapter, an experimental evolution approach was applied in a bid to improve PpR24's virulence to aphids and to investigate whether biofilm formation can evolve as a means to improve bacterial persistence on a plant, thus reducing the number of applications needed to a crop. Ten independent lineages of PpR24 were passaged through aphids via aphid diet sachets to improve aphid virulence and another ten lineages through King's broth medium B microcosms to evolve biofilm growth *in vitro*. However, no attempt was made to understand the underlying changes between observed phenotypes (which shall be explored in Chapter 4).

3.4.1 Aphid virulence

Applied experimental evolution serial passage approaches are not always successful in evolving a desired trait, such as the unsuccessful attempt to evolve nitrogen fixation in symbiotic plasmid-carrying *R. solarnacearum* [146]. In the current study, after the ten passage cycles through aphids, no improvement to virulence was seen in any of the ten isolates derived from the wild-type PpR24. It is possible that ten passages was an insufficient number of cycles to allow any virulence mutations to evolve. Unlike the single, microcosm environment of the biofilm passages, isolates passaged through aphids were exposed to different environments. By alternating the bacteria through the aphids (where bacteria would have to contend with competition from gut symbionts and the aphid immune system) and a plate environment, it is possible that an inadequate selection pressure acted on the isolates to improve aphid virulence over the passages. Previous studies following a similar methodology to enhance the virulence of entomopathogenic fungi against target pest insects also proved unsuccessful in improving virulence [192, 193]. For example, no significant change was observed in *B. bassiana* virulence to malarial mosquitoes after ten passage cycles [159]. Therefore an insufficient number of passages may be the reason for no observed change in PpR24 isolate virulence [159].

Another factor that may affect the evolution of virulence in PpR24 is the life-cycle of the bacterium and how it is transmitted from the aphid host [135].

The trade-off hypothesis between virulence and transmission assumes that an organism of high growth and virulence may kill its host before it has time to spread to other hosts, whereas a species with slow growth may have a high transmission to another host. An equilibrium between virulence and transmission is needed for the parasite to maximise its fitness. It is possible that the wild-type PpR24 is already at this equilibrium and a higher virulence would adversely affect the bacterium's fitness [194, 195, 159]. Furthermore, the Paliwal study found the wild-type PpR24 reproduced in the aphid gut to a population of $2x10^7$ CFU/aphid, therefore the population of aphid-passaged isolates may not reach high enough titres within the aphid gut to allow for beneficial mutants to evolve.

3.4.2 Biofilm formation

Attempts to evolve biofilm formation were more successful than the virulence passages. After ten passages, one isolate out of ten lineages evolved the ability to form consistently strong biofilms at the air-liquid interface. Isolate PpR24b4 formed significantly stronger biofilm at the air-liquid interface than all other passaged isolates. As seen in similar studies conducted with SBW25 Wrinkly Spreader, forming a biofilm may provide mutants with a fitness advantage by accessing the oxygen-rich surface of a microcosm, so more resources are available for growth, allowing the mutants to proliferate through the system [177, 178]. PpR24b4's biofilm formation appears to have established in the system at passage 7, whereas other isolates under the same selection exhibited more stochastic and inconsistent biofilm growth over the ten passages. It is possible that over the week long period each passage was left to grow, biofilm structures became too thick and dense to be self supported and cellular matter may have sunk to the bottom of the microcosm, thus avoiding any forms of quantitative measurement. However, isolate PpR24b4's attachment strength to the glass microcosm walls was not significantly stronger than that of the other biofilm isolates.

By the final passage, isolate PpR24b2 and PpR24b5 also appeared to form a biofilm at the air-liquid interface. These biofilms were unable to support any significant mass before breaking or sinking. It is possible that in these biofilms, 'cheats' had established in the system. Such biofilm cheats are cells that take advantage of the communal goods and services supplied by cells in

the biofilm aggregation, without contributing to the formation of the biofilm themselves [163]. This enables the cheats to spend their energy in more selfish ways, such as reproduction, enabling these undesirable mutants to proliferate through the system [163].

The evolution of PpR24 from planktonic to biofilm-former appears to be at least a two stage process, with passages first forming a opaque, yellow broth solution with a weak biofilm at the air-liquid interface that cannot support much/any weight. When a strong biofilm is formed, as in PpR24b4, the yellow colouration is lost which may indicate a loss or reduction of siderophore production. After peaking at approximately the sixth passage, the biofilm strength of the PpR24b4 isolate appears to plateau. This may be due to a variety of limiting factors, such as nutrient availability in the microcosm, which means it is unable to develop any further [196].

3.4.3 Trade-off analysis

No significant phenotypic changes were observed between the aphid passaged bacteria and wild-type PpR24. However, several trade-offs were observed to be associated with the biofilm passaged isolates. Although well adapted to growth in static broth conditions, biofilm isolates performed less well than the wild-type and aphid-passaged isolates when grown on agar plates or in a shaken environment. When grown on an agar plate, the wild-type and aphid-passaged colonies were round, approximately 2mm in diameter and yellow in colour with a blurred boundary at the edge of the colony. In contrast, PpR24b4 formed smaller, circular, dot-like colonies that were milky in colour strikingly different conformation to colonies formed by the SBW25 WS mutant, therefore it is possible the biofilm mutation evolved in different pathways. Other studies have observed similar trade-offs in growth, such as the *Pseudomonas fluorescens* SBW25 WS mutant which had significantly poorer growth in shaken broth conditions and when grown on a plate when compared to static microcosms [177].

The most important trade-off observed between the two selection lineages was that biofilm-passaged isolates were significantly less virulent than those of the aphid passaged isolates. The statistically strong biofilm forming isolate PpR24 was significantly less virulent to aphids in 48 hours than the wild-type and aphid passaged strains. This loss in virulence may in part be due to the

biofilm-passage isolates reduced growth in non-static condition. Evidence of reduced growth on agar plates and in shaken broth conditions may be extrapolated to imply poor adaptation and fitness in the aphid gut environment. Biofilm isolate populations may not have reached the lethal concentrations required to cause aphid mortality within the 48 hour duration of the experiment. Furthermore, poorer growth may entail the biofilm isolates to be less competitive in the aphid gut environment. The aphid gut microbiome plays host to a variety of endosymbionts, many with anti-pathogenic properties. For instance, *Regiella insecticola* provides its aphid host with protection against pathogens and parasitoids [197]. It is possible that the reduced growth of the derived biofilm isolates inhibits their ability to compete with the aphids' endosymbionts, thus reducing aphid virulence.

The loss of virulence in biofilm forming isolates may also be associated with their reduced motility in swimming and swarming agar. In many pathogens, motility is considered an important factor contributing towards virulence [180]. Flagellum-driven motility enables pathogens to move to more favourable environments and find resources and loss of flagella has been correlated with a loss of virulence [162], for instance *Pseudomonas aeruginosa* M-2 mutants with no polar flagellum lost their virulence [184].

However, although biofilm mutants had reduced motility in swarming and swimming soft agars, regardless of whether a biofilm was present in at the air-liquid interface of the microcosm, no isolates exhibited a total loss of motility. Indeed, motility can be key in the initial attachment stages of biofilm formation [162]. Therefore, the reduction in cellular movement may be due to the reduced growth of the isolates on agar, rather than due to a loss of flagellum. Furthermore, upon establishing as a mature biofilm colony, cells adjust to their sedentary lifestyle and lose their energy-demanding flagella, instead increasing EPS production [162, 180]. As the final isolates taken as stocks for PpR24b4 were from a mature biofilm colony, this may also account for its reduced motility.

Biofilm formation did not improve bacterial colonisation of sweet pepper crops as hypothesised. This study found that after three weeks of growth on pepper plants, no significant difference in bacterial populations recovered from the plants was found between the wild-type and either aphid-passaged or biofilm-passaged final evolved isolates. Although PpR24b4 was capable of forming a strong biofilm in static microcosms, it cannot be concluded from

this study that the PpR24b4 isolates recovered from the phylloplane were growing in biofilm aggregations. However, having too successful a biofilm on the phyllosphere may prove adverse to the plant survival, potentially impeding vital leaf functions by blocking stomata which would prove detrimental to the plant and possibly result in higher yield loss if applied to a crop.

3.5 Conclusion

An experimental evolution approach was successful in evolving biofilm-forming mutants *in vitro* but no significant improvements were evolved regarding aphid virulence. Significant trade-offs were observed in biofilm-forming isolates, which exhibited poorer growth and motility than the wild-type PpR24 and aphid-passaged isolates. Furthermore, no significant improvements to plant colonisation was seen in either the derived biofilm or aphid-passaged isolates. Overall, in spite of attempts to improve PpR24's performance as a potential biocontrol agent, when compared to evolved PpR24 isolates, the wild-type PpR24 remains the best candidate for an aphid biopesticide.

Chapter 4

Analysis of genomic changes

4.1 Introduction

The experimental evolution passages conducted in Chapter 3 resulted in significant phenotypic differences between the wild-type PpR24 and the derived biofilm-passaged isolate PpR24b4. PpR24b4 formed statistically stronger biofilms in a broth environment than the wild-type PpR24 and other passaged isolates. However, biofilm production evolved at an apparent trade-off with bacterial growth, isolate motility and, in terms of use as an aphid biocontrol agent, the most important change observed in PpR24b4 was its reduction in aphid mortality. This chapter uses whole genome sequencing to investigate the underlying genetic mechanisms that may be responsible for the phenotypic changes observed in the derived isolates from Chapter 3.

4.1.1 Experimental evolution and whole genome sequencing

Experimental evolution coupled with whole genome sequencing offers a comprehensive and versatile approach to understanding the underlying processes of evolution. Whole genome sequencing can elucidate the genomic factors responsible for phenotypic and fitness changes that evolve over the course of experimental passages [198, 147]. By using a reference genome of a wild-type ancestor isolate, derived isolates evolved under selection conditions can be sequenced and compared to the reference genome to identify mutations [199]. For example, in a study investigating streptomycin resistance in the probiotic *Lactobacillus plantarum* ATCC14917, isolates were grown in a strep-

tomycin environment over 25 days and evolved a high level of resistance to streptomycin. Whole-genome sequencing revealed evolved isolates possessed five mutated genes when compared to the ancestor strain, three were single nucleotide polymorphisms (SNPs) and two were structural variants, with one SNP in the gene encoding small subunit ribosomal protein 12 (S12) concluded to be the likely factor behind streptomycin resistance [200].

As well as a tool for examining the root of specific traits, whole genome sequencing can be applied to unravel broader questions surrounding evolutionary dynamics and the driving factors and processes of mutation and adaptation [198, 134]. The long-term evolution experiment with *E. coli* lends itself particularly well to such studies, such as investigations into which areas of the genome are targets for selection and parallel evolution [198, 134].

4.1.2 Hybrid assembly

Whole genome sequencing offers an in-depth approach to variant detection, such as substitutions, In-Dels and single nucleotide polymorphisms (SNPs), detecting more mutations with a greater accuracy and with no dependence on prior knowledge of the target genome, unlike alternative approaches, such as candidate gene sequencing and microarray-based analysis [198, 201, 202]. Illumina platform sequencing has been widely used to study bacterial genomics due to reads having a high accuracy, with a low (<1% per-base) error rate, and relatively low cost. However, Illumina sequencing utilises short DNA fragments of 500 base-pairs (bp) or less, resulting in fragmented contiguous sequences from which it can be hard to construct an accurate and complete genome [203]. Long read approaches to sequencing, such as Pacific Biosciences and Oxford Nanopore Technologies, are more expensive than Illumina reads and less accurate, with a 5-15% per-base error rate, but are able to sequence fragments of 10 kbp or longer for complete genome assemblies.

Hybrid assembly uses a combination of long and short reads to achieve accurate, complete genome assemblies [204, 203]. Long reads provide a scaffold upon which the accurate short reads are assembled, resulting in improved resolved genome quality compared to either a short-read only or long-read only assembly. Hybrid assembly has been used across a range of taxa to produce a more accurate genomes, such as of the clown anemonefish, *Amphiprion ocellaris* [204], as well as prove a vital tool in elucidating the genetic basis behind

traits. For instance, hybrid assembly was used to identify the factors responsible for anti-fungal and plant growth promoting properties of the bacterium *Paenibacillus pasadenensis* strain R16 [205].

4.1.3 Aims of this Chapter

In the well-studied case of the *Pseudomonas fluorescens* SBW25 biofilm-forming Wrinkly Spreader (WS) phenotype, a mutation in the methylesterase response regulator, *wspF*, of the Wsp operon, was responsible for biofilm formation [206]. Mutations in *wspF* that rendered it non-functional had a knock-on effect in the over activation of WspR, causing the over production of c-di-GMP and adhesive substances such as a cellulic polymer that results in the WS phenotype [207]. To investigate this mutation, 26 independently evolved lineages of WS genotypes, seeded from the same ancestral 'smooth strain of *P. fluorescens* SBW25, were placed under selection for 5 days in separate microcosms. At the conclusion of the experiment, 13 of the 26 isolates all contained simple nucleotide changes, either transitions, transversions, or short deletions, in *wspF* that had varying effects on isolate fitness [207, 177]. The fact that so many independent populations evolved parallel genetic changes suggests that mutations in *wspF* are indicative of adaptive evolution to the broth environment [207]. SBW25 *wsp*-like operons are also present in related pseudomonads, such as *P. fluorescens* Pf0-1, *P. putida* KT2440, and *P. syringae* pv *tomato* DC3000 [206], therefore it is possible that similar, simple mutations in the Wsp system are responsible for the biofilm phenotype of *P. poae* PpR24b4.

In this chapter, a hybrid assembly approach was employed to identify mutations in the evolved isolate PpR24b4 that differed from the wild-type PpR24 and aphid-passaged PpR24a1, which may account for the biofilm phenotype. The end-point isolate from the final evolution passage isolates of PpR24b4 and PpR24a1 were sequenced to identify fixed mutations in the evolved isolate populations [132].

4.2 Materials and Methods

Whole genome sequencing was conducted on three *P. poae* strains: the wild-type PpR24 and two final passage isolates, aphid-passaged PpR24a1 and biofilm forming isolate PpR24b4. PCRs and DNA extractions were conducted as in Chapter 2, Sections 2.7-2.10.

4.2.1 Illumina and Nanopore Sequencing and hybrid assembly

MicrobesNG conducted combined Illumina and Nanopore Sequencing and hybrid assembly. Strains were prepared in accordance with the enhanced genome service protocol.

Single colonies of PpR24, PpR24a1 and PpR24b4 were mixed in 200μl of sterile 1xPBS, 100μl of which was used to inoculate 50ml of sterile KB. The remaining 100μl suspension was streaked out onto a KA plate to determine whether the culture was pure. The samples were incubated until the upper exponential phase, centrifuged for 10 minutes at 500xg and the supernatant discarded until a combined wet weight of 300mg pelleted cells was achieved. The pelleted cells were re-suspended in 500μl of the cryopreservant liquid from the barcoded bead tube supplied by MicrobesNG. The tubes were sealed with parafilm and sent at room temperature to MicrobesNG.

Three beads were washed with extraction buffer containing lysozyme and RNase A, incubated for 25 min at 37°C. Proteinase K and RNaseA were added and incubated for 5 min at 65°C. Genomic DNA was purified using an equal volume of SPRI beads and re-suspended in EB buffer. DNA was quantified in triplicates with the Quantit dsDNA HS assay in an Ependorff AF2200 plate reader. Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturers protocol with the following modifications: two nanograms of DNA instead of one were used as input, and PCR elongation time was increased to 1 min from 30 seconds. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system. Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche light cycler 96 qPCR machine. Libraries were sequenced on the Illumina HiSeq using a 250bp paired end protocol. Reads were adapter trimmed using Trim-

momatic 0.30 with a sliding window quality cutoff of Q15. *De novo* assembly was performed on samples using SPAdes version 3.7, and contigs were annotated using Prokka 1.11.

4.2.2 Variant Calling Analysis

Variant calling was conducted by the Reading University bioinformatician, Dr Salehe. The hybrid assembled genomes provided by MicrobesNG consisted of annotation files in gbk and gff formats in addition to the fasta (.fna) format. Overall, 6532916 short reads and 8883 long reads were aquired. The genomes were *de novo* assembled by Unicycler tool (MicrobesNG). The short raw reads were quality checked for sequencing and library prep errors using FastQC v0.11.8 [208]. SNIPPY v4.4.0 [209] pipeline in the command line mode was used for variant calling and each sample read for each strain was mapped against the wild-type reference. The SNIPPY pipeline was specifically designed for calling variants in haploid organisms. The variants were annotated by using the snpEff v4.3t, which was part of the SNIPPY pipeline.

4.2.3 Gene sequence alignment

Sanger sequencing was conducted by Eurofins for *cheb* and *barA* of each isolate. Sequences were trimmed and consensus sequences were produced using BioEdit software. Forward and reverse sequences were aligned to form a consensus sequence. Consensus sequences of the derived isolates were Blast [210] using the NCBI website in comparison with the wild-type and similarity to the wild-type nucleotide sequence observed.

4.2.4 Basic Local Alignment Search

BLASTX [210] analysis was performed on the FASTA sequences of the wild-type and evolved PpR24 isolates to corroborate the findings of the variant calling analysis. Sequences were Blasted against the non-redundant protein sequences (nr) database, using BLOSUM62 matrix with an expect threshold of 10, word size of 6 and an e-value cut-off of 10^{-3}. To identify the locations of amino acid replacements in context of the protein, sequences were BLASTed against the UniProtKB reference proteome plus Swiss-Prot database in UniProt, with an E-threshold of 10.

4.3 Results

4.3.1 Variant calling analysis

Stark phenotypic differences were observed between the wild-type PpR24 and isolate PpR24b4, with PpR24b4 exhibiting biofilm formation, poorer growth and poorer aphid virulence compared to the wild-type. Hybrid assembly of Oxford Nanopore long reads and Illumina short reads were used to resolve complete genomes of the isolates and variant calling conducted using Unicyler pipeline in a bid to elucidate the genomic factors behind the changes in phenotype. Even though no significant differences were observed in phenotype between the wild-type PpR24 and aphid-passaged isolate PpR24a1, PpR24a1 was also investigated for mutations as the trade-off analysis may not have been sufficiently comprehensive to detect divergent isolates.

After sequencing final passage isolates, in regions conserved across all three isolates (PpR24, PpR24a1 and PpR24b4) only two single nucleotide variants were identified that differed from the reference wild-type *P. poae* PpR24 genotype. Both SNPs occurred on the chromosome of the biofilm passaged isolate, PpR24b4. One mutation occurred at the genomic position 728018 in the gene *cheB_1* (*wspF* in Pseudomonads) where a point mutation changed a T to an A that resulted in a serine being replaced with threonine (Ser159Thr). Interestingly, although occurring on the same gene as the *wspF* WS mutations thought to be responsible for biofilm formation in SBW25, the specific amino acid change from serine to threonine was not one of the 13 mutations recorded in Wrinkly Spreader mutants [207]. The second mutation occurred at 3010602 in the gene *barA_3* (*gacS* in Pseudomonads) where a G became T, resulting in a serine changing to isoleucine.

4.3.2 Gene sequence alignment

Sanger sequencing and sequence alignment in BioEdit was conducted to confirm the findings of the variant calling analysis. The results corroborated with the variant calling analysis. Consensus alignments of isolate PpR24a1 and the wild-type PpR24 had a 100% similarity for both *cheB* and *barA* sequences. However, the forward sequence for the wild-type bacteria *barA* gene failed to align, therefore only the reverse sequence was used in the comparison of the consensus *barA* sequences for PpR24a1 and PpR24b4.

For *cheB*, BLAST results between the sequences under investigation found a 99% similarity between the gene sequences of PpR24 and the wild-type PpR24. A single point mutation was observed at location 290 on the gene. BLAST results found 99.73% similarity between the *barA* sequences for PpR24b4 and the wild-type, with a point mutation at location 110. However, the sequence alignments did not match for the forward and reverse sequences on point mutation in *barA*. Although variant calling found a G on the wild-type became T in PpR24b4, BioEdit alignment found a discrepancy in the nucleotides base pairs at the point mutation site in PpR24b4 as the forward sequence read C and the reverse A.

4.3.3 Investigation into gene function

To elucidate gene function, wild-type and evolved isolate sequences were searched against the NCBI BLAST non-redundant database, using BLASTX algorithms. The best hit results for *barA* indicated it matched a HAMP domain-containing protein response regulator in *Pseudomonas fluorescens* and several *Pseudomonas* species. For *cheB*, the best hit results suggested the gene matched the chemotaxis response regulator protein, glutamate methylesterase in *Pseudomonas lactis* and a range of other *Pseudomonas* species (the four best BLAST hits for each gene can be found in Table 4.1).

Table 4.1: NCBI BlastX results for *barA* and *cheB*.

Organism	NCBI Accession Reference	Query Cover	E value	% Identity	Definition
barA					
Pseudomonas fluorescens	WP_155718429.1	100%	1e-78	100.00%	HAMP domain-containing protein (partial)
Pseudomonas sp. NFPP02	SFX32716.1	100%	4e-78	100.00%	HAMP domain-containing protein
Pseudomonas sp.	WP_060765691.1	100%	4e-74	100.00%	Multi-species: response regulator
Pseudomonas lactis	WP_153475311.1	100%	5e-74	100.00%	Response regulator
Pseudomonas fluoresecens A506	AFJ58380.1	100%	2e-73	99.19%	Sensor protein GacS
cheB					
Pseudomonas lactis	WP_094774784.1	99%	1e-112	99.40%	Chemotaxis response regulator protein-glutamate methylesterase
Pseudomonas sp.	WP_130174755.1	99%	3e-112	98.80%	Multi-species: chemotaxis response regulator protein-glutamate methylesterase
Pseudomonas sp.	WP_047712720.1	99%	4e-112	99.40%	Multi-species: chemotaxis response regulator protein-glutamate methylesterase
Pseudomonas fluorescens	WP_089041286.1	99%	9e-112	98.80%	Chemotaxis response regulator protein-glutamate methylesterase
Pseudomonas sp. L13	WP_161906685.1	99%	3e-111	98.19%	Chemotaxis-specific protein-glutamate methyltransferase CheB

An assessment as to whether the sequences detected were orthologous to the wild-type and biofilm-phenotype sequences via reciprocal BLAST was conducted using the best hit BLASTX results (Table 4.1) for the sequences of interest and the reference *P. poae* genome. The reciprocal BLAST for *cheB* in *P. lactis* identified a chemotaxis-specific methylesterase as the best match (Accession AGE24666.1, 100% query cover, E value 2e-100, percentage identity 87.35%), with the fourth best match a protein-glutamate methylesterase belonging to the CheB family (Accession AGE25786.1, 46% query cover, E value 3e-05, percentage identity 31.17%). As these hits retain the same function as the original sequence, it can be assumed they are orthologues, rather that paralogues [211]. Similarly for the *barA* sequence, the sensor protein GacS was the best match (Accession AGE26284.1, 100% query cover, E value 6e-74, percentage identity 95.12%). As GacS is the *Pseudomonas* homologue of BarA [212], this suggests that the genes are orthologous.

To investigate the amino acid changes in context of the wider protein, UniProt alignments were created using the highest matched, reviewed Swiss-Prot protein and the best hit from the BLASTX against the NCBI BLAST non-redundant database. The best hit UniProt swiss-reviewed sequence for *cheB* was Q4KHL8 (CHEB1_PSEF5) *Pseudomonas fluorescens* (strain ATCC BAA-477 / NRRL B-23932 / Pf-5), a protein-glutamate methylesterase/protein-glutamine glutaminase in the gene *cheB_1* with an E-value of 1.5e-86 and 82.5% identity. In the PpR24b4 biofilm mutant, the amino acid replacement occurred in DOMAIN 143-336 of CheB-type methylesterase at the active site, ACT_SITE 159-159 (Figure 4.2), therefore it is possible that the SNP could affect the protein structure and activity.

In the case of *barA*, protein alignments were made with the best reviewed swiss-prot GACS_PSEPH - Sensor histidine kinase GacS (Q9F8D7) from *Pseudomonas protegans*, which had an E-value of 2.7e-16 and identity of 87%. When aligned against *Pseudomonas protegens* (strain DSM 19095 / LMG 27888 / CHA0) the mutation in GacS, the Pseudomonad homologue to BarA, occurred in a conserved region of the HAMP DOMAIN 192-244 of the Sensor histidine kinase CHAIN 1-917 of GacS, which corroborated with the BLASTX result, where the best match in *P. poae* was the sensor protein GacS (Figure 4.3).

Figure 4.1: CheB protein alignment. Amino acid sequence alignment conducted in UniProt, where 'wt' is the wild-type PpR24 sequence, 'PpR24b4' the biofilm phenotype, 'Q4KHL8 CHEB1_ PSEF5)' the best reviewed reference match in the UniProt BLASTX database *Pseudomonas protegens* (strain DSM 19095 / LMG 27888 / CHA0), and 'WP_ 094774784' the best match after BLASTX chemotaxis response regulator protein-glutamate methylesterase in *Pseudomonas lactis*. The amino acid replacement occurred at an active site in the CheB methylesterase domain, where serine was replaced with threonine.

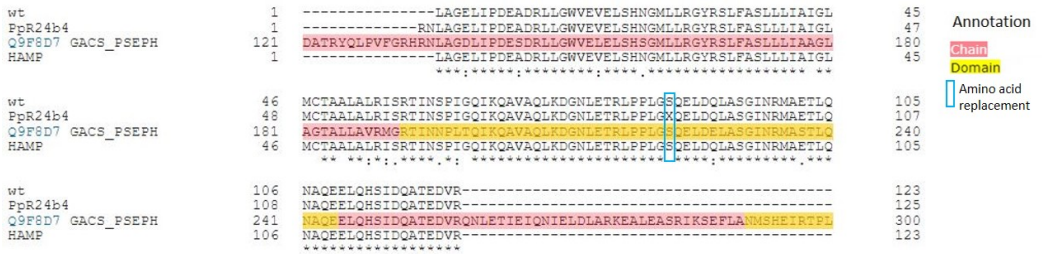


Figure 4.2: BarA protein alignment. Amino acid sequence alignment conducted in UniProt, where 'wt' is the wild-type PpR24 sequence, 'PpR24b4' the biofilm phenotype, 'Q9F8D7 GACS_ PSEPH' the best reviewed reference match in the UniProt BLASTX database *Pseudomonas fluorescens* (strain ATCC BAA-477 / NRRL B-23932 / Pf-5), and 'HAMP' the best match after BLASTX HAMP domain-containing protein from *Pseudomonas fluorescens*. The amino acid change in isolate PpR24b4 was present in the HAMP domain, where serine potentially became isoleucine.

4.3.4 The genomic context of *cheB* and *barA*

To further substantiate the identification of *cheB* and *barA*, the context of the genes in relation to the wider genome was investigated to assess whether

nearby genes matched their expected pathways (Figure 4.3 and Table 4.2).

Several genes neighbouring *cheB* have been associated with the Che system of *E. coli*, homologous to the Wsp chemosensory pathway of Pseudomonads. The methyl-accepting chemotaxis protein, *mcpB*, is homologous to *wspA*, *cheW_ 1*, a protein in the Che pathway homologous to *wspB* and *wspD*, and the methyltransferase *wspC* [213].

As *barA*, a histidine sensor kinase, forms a two-component signal transduction system with *urvY* [214], it was expected that *urvY* would be identified as a neighbouring gene. This was not the case (Figure 4.3 and Table 4.2), although the histidine kinase *pfeS* (homologous to the sensor histidine kinase EnvZ protein in *E. coli*), was identified along with its counterpart, *ompR_ 4*. Together the two form two-component signal transduction system to regulate osmotic stress response [215].

Table 4.2: Identified genes neighbouring *cheB* and *barA*.

Gene	Function
barA	
comEC_1	ComE operon protein 3
comEC_2	ComE operon protein 3
pfeS	Sensor protein PfeS
ompR_4	Transcriptional regulatory protein OmpR
ldhA	Lactate dehydrogenase
resA_1	Thiol-disulfide oxidoreductase ResA
arsC_2	Arsenate reductase
cheB	
mcpB	Methyl-accepting chemotaxis protein McpB
cheW_1	Chemotaxis protein CheW
wspC	Probable biofilm formation methyltransferase WspC
frzE	Gliding motility regulatory protein
cph2_2	Phytochrome-like protein cph2
prfB	Peptide chain release factor RF2
lysU	tRNA ligase%2C heat inducible
rcdA	HTH-type transcriptional regulator RcdA

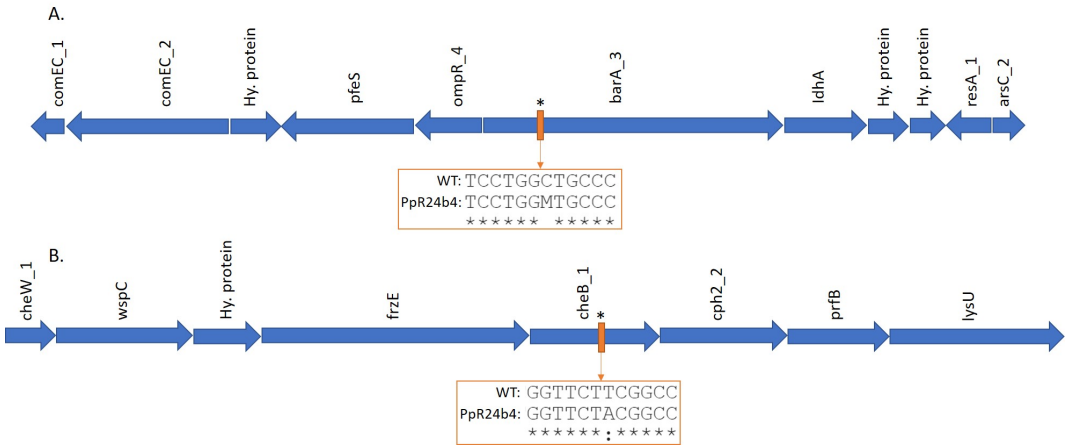


Figure 4.3: The genomic context of *cheB* and *barA*, where 'wt' is the wild-type PpR24 sequence and 'PpR24b4' the biofilm phenotype. A point mutation occured at location 3010602 in the gene *barA_3*, and a second at genomic position 728018 in the gene *cheB_1*. 'Hy. protein' stands for hypothetical protein.

4.4 Discussion

Elucidating genetic variation is vital in understanding phenotype and adaptive evolution. In this study, hybrid assembly with variant calling analysis of the wild-type PpR24 and final passage isolates PpR24a1 and PpR24b4 identified two single nucleotide polymorphisms present on the PpR24b4 genome, one in the gene *cheB* and the other in *barA*. No genomic differences were observed between the wild-type and PpR24a1 isolates. Both *cheB* and *barA* are genes associated with bacterial motility and biofilm formation, therefore it is possible that one, or both of these mutations is responsible for the evolved biofilm properties of PpR24b4.

4.4.1 *cheB* (*wspF*)

In *E. coli*, CheB is a methylesterase protein in the chemotaxis signalling pathway CheIV, a key pathway in swarming behaviour [188]. Along with the proteins CheA and CheY, CheB controls the direction of flagellum rotation, switching the rotation from clockwise to the default state of counter-clockwise movement. This allows the bacterium to move towards or away from a stimulus [213, 188]. In chemotaxis, directional movement occurs in response to a chemical stimulus. Chemical stimuli bind to methyl-accepting chemotaxis proteins (MCPs) in the bacterial membrane via ligand binding, which triggers a change in the MCP that modulates the autophosphorylation of an associated histidine kinase (CheA). CheA-P phosphorylates the response regulator CheY, and CheY-P binds to the flagellar motor, causing the flagella to rotate clockwise. Concurrently, CheA modulates the phosphorylation state of CheB through phosphotransfer. CheB-P is active as a methylesterase and acts in conjunction with constitutively active methyltransferase (CheR) to adjust the methylation state of glutamate residues on MCPs. By shifting the methylation state, the flagella rotation is restored to the counter-clockwise direction [207, 213](Figure 4.4).

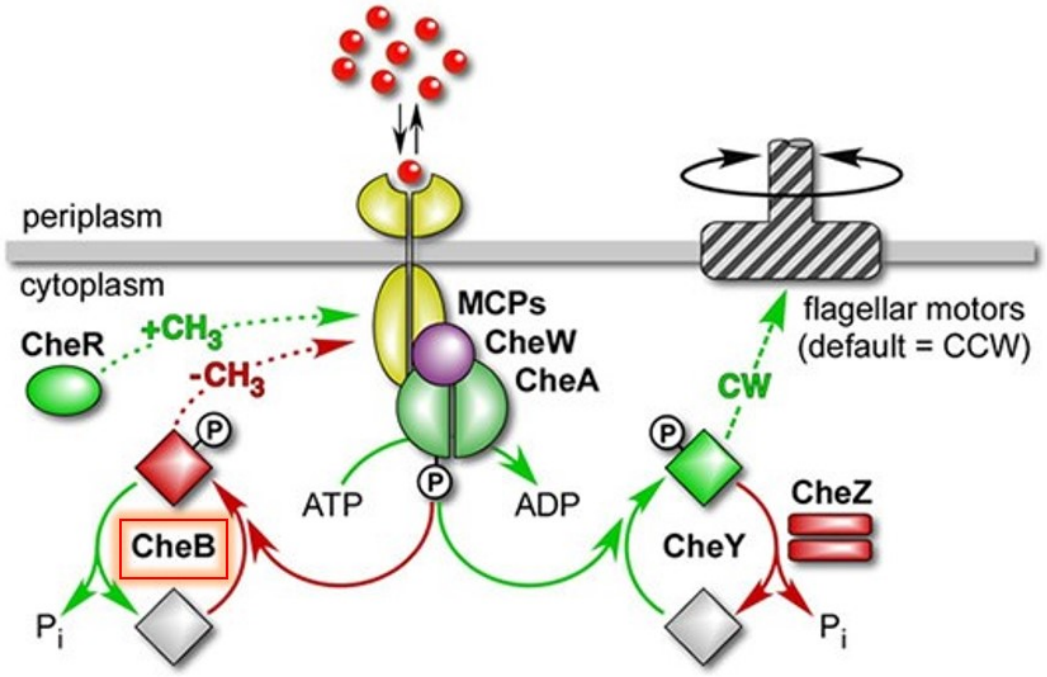


Figure 4.4: CheB in *E. coli* chemotaxis. CheB removes methyl groups, returning the flagellar motor to its pre-activated state of counter-clockwise rotation (image adapted from [216]).

In Pseudomonads, the WspF protein is a methylesterase homologue of CheB involved in reacting to chemotactic stimuli [213]. The Wsp chemosensory pathway, homologous to the CheIV pathway, has been shown to regulate numerous factors associated with biofilm formation and the Wrinkly-Spreader phenotype, such as the SBW25 *wss* operon responsible for the production of a cellulose-like polymer that makes up the main structure of the biofilm [217]. The Wsp pathway is highly conserved across *Pseudomonas* genomes [207, 218, 213, 206] and is composed of seven open reading frames, *wspABCDEFR*, over approximately 8.5 kb [207](Figure 4.5).

As mentioned in Section 4.1.3, WspF is responsible for the activation of the response regulator, WspR which possesses a GGDEF domain responsible for the formation of the important intracellular signalling molecule, cyclic diguanylate (cyclic di-GMP/c-di-GMP). Cyclic di-GMP plays a central role in coordinating the transition between a motile or sessile life-style in several bacterial species by regulating a suite of genes involved in exopolysaccharide (EPS) production, cellulose synthesis, TypeIV pili and motility [219, 213, 220, 221]. A loss-of-function in WspF leads to the constitutive activation of WspR and

the over-production of c-di-GMP. High-levels of c-di-GMP in a cell is thought to be a causal factor in biofilm formation, causing a repression of genes associated with virulence and a free-floating planktonic state, and stimulating cell aggregation and biofilm formation. For example, in *P. aeruginosa*, biofilms are estimated to contain on average 75110 pmol of c-di-GMP per mg of total cell extract, whereas planktonic cells contain less than 30 pmol mg^{-1} [222].

Mutations in WspF of *P. fluorescens* SBW25 cause dramatic changes in the bacterial phenotype compared to the wild-type, resulting in a Wrinkly-Spreader (WS) phenotype [207]. Similarly in *P. aeruginosa* PAO1, disruption of *wspF* resulted in cellular aggregation and a wrinkled appearance when grown on agar [223]. The well-studied Wss operon in *P. fluorescens* SBW25 is a vital contributing factor to the WS phenotype and regulated by WspR. The Wss operon is responsible for the production of a partially acetylated cellulose polymer essential to the SBW25 Wrinkly Spreader's biofilm structure and wrinkled appearance [207, 220]. As well as forming cell aggregations, WS mutants are less motile than the wild-type SBW25 [207, 223, 188]. An important target of c-di-GMP produced in the Wsp pathway is the transcriptional regulator, FleQ, which downregulates biofilm-associated genes and promotes flagellar genes [218]. At high concentrations of c-di-GMP, FleQ is inhibited, causing reduced expression of flagellar genes which initiates cell aggregation and biofilm formation [218, 213, 222].

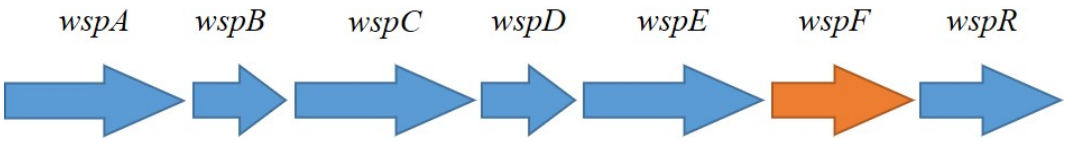


Figure 4.5: Organisation of genes in the Wsp chemosensory system (adapted from [213]). WspA acts is a membrane-bound methyl-accepting chemotaxis protein. WspB and WspD act as scaffold proteins. WspC is a cheR-like methyltransferase. WspE is a hybrid histidine kinase-response regulator. WspF acts as cheB-like methylesterase (orange to indicate the presence of the mutation in this study). WspR is a response regulator with a GGDEF domain, which plays an important role in the production of the intracellular signalling molecule cyclic diguanylate (c-diGMP) [207, 213].

4.4.2 *barA* (*gacS*)

The gene *barA* is responsible for the histidine sensor kinase, BarA, in the two-component signal transduction system (TCS) of *E. coli*, where UvrY acts as its counterpart response regulator [214]. In *Pseudomonas* species, the homologue of this system is the GacS/GacA TCS, where GacS is homologous to BarA as a membrane-bound sensor kinase, and GacA is a cytoplasmic transcriptional response regulator [224, 212]. The GacS/GacA system regulates secondary metabolite and extracellular protein production and is highly conserved in Gram-negative bacteria [225]. Along with other TCSs, the GacS/GacA system has been shown to play an important role in the coordination of the transition between a motile and biofilm lifestyle.

The autophosphorylation of GacS stimulates the transfer of a phosphate to GacA, via the GacS/GacA phosphorelay system. Once activated, GacA mediates the transcription of small RNA molecules, such as RsmX, RsmY, RsmZ, and RsmA protein [226], which regulate the expression of a wide range of genes, some involved in secondary metabolism [227]. The GacS/GacA system has been well studied in Pseudomonads [222]. In *P. aeruginosa*, high levels of RsmA have been found to promote a planktonic, virulent lifestyle by the upregulation of Type III secretion systems, whereas low levels of RsmA results in the production of biofilm determinants [219].

In Gram-negative bacteria the GacS/GacA system has been associated with biofilm formation, quorum sensing, siderophore production, motility, volatile organic compound production, and bacterial virulence to plants, fungi and animals [224, 228, 212, 229, 230] (Figure 4.5). Gac is an acronym for Global Activator of antibiotic and Cyanide synthesis [231] and many antibiotic and virulence exoproducts in fluorescent Pseudomonads are dependent on the GacS/GacA regulatory system, such as hydrogen cyanide, pyocyanine, phenazines, lipopeptides, and exoproteases [114, 231, 212, 115]. Furthermore, the GacS/GacA system has been seen to contribute to the regulation of the anti-metabolite toxin phaseolotoxin produced by *Pseudomonas syringae* pv. *phaseolicola* [232], and the insecticidal toxin complex component TccC in *Pseudomonas taiwanensis* [121].

The role of the GacS/GacA system in siderophore production is unclear. Siderophores, such as pyoverdine and pyochelin, are important secondary metabolites produced by microorgansims to acquire iron(III) from an environment

[233]. In *P. fluorescens* and *P. chlororaphis*, a defect in *gacS-gacA* increased production of siderophores, including pyoverdine [233]. GacS was also observed to repress traits such as pyoverdine-like siderophore formation in *P. chlororaphis* O6 and P23 [229]. Pyoverdine is responsible for the yellow-green pigment in fluorescent Pseudomonands [234], therefore it is possible that a mutation in *gacS* may affect siderophore regulation, and as a consequence, pigment production, resulting in the loss of pigmentation seen in the strong biofilm forming isolate.

Mutations in *gacS/gacA* have been seen to result in a reduction or total loss of biocontrol abilities in plant growth promoting bacteria, and a loss of virulence in plant and animal pathogens [235, 236]. Mutants have also been seen to have an altered colony structure and changes in motility [237]. Variants of *P. fluorescens* F113 with *gacS/gacA* mutations had significantly increased swimming motility than the wild-type [238]. Inactivation of the *gacS* gene in *Pseudomonas aeuriginosa* PA14 resulted in hypermotility and a reduction in biofilm formation. However, biofilms of these sensor kinase deficient mutants gave rise to small colony variants when cultured, which were less motile and showed a hyper-biofilm-forming phenotype [228], similar traits to PpR24b4 in the current study.

It is possible that the missense mutation in GacS may be a compensatory mutation to overcome the fitness cost of transitioning to a broth environment. It has been proposed that the GacS/GacA TCS may serve as a contingency loci for environmental adaptation, where a high incidence of mutations may allow for phenotype switching [239]. Phenotype switching is responsible for the regulation of a variety of secondary metabolites, the production of which may no longer be necessary in a broth environment. Such compensatory evolution has been observed in plasmid acquisition of *P. fluorescens* SBW25, involving the plasmid pQBR103 that confers mercury resistance [240, 239]. Plasmid containing bacteria were experimentally evolved in six concentrations of toxic mercuric ions, Hg(II), and after 450 generations, mutations were present in either *gacA* or *gacS* of approximately 80% of plasmid carrying clones but no such mutations were observed in plasmid-free clones. It is possible that the evident mutations in *gacS* are the result of phenotypic switching as the bacterium adapts to the broth environment and non-essential secondary metablolite production, such as aphicidal toxins, are downregulated to balance the fitness cost of adapting to a novel environment.

A subsequent study of the current project, conducted by Masters student Paolo Capano, used the 'fossil record' of the evolutionary passages conducted in Chapter 3 to sequence the genes of interest, *wspF* and *gacS*, in isolate PpR24b4 and isolate PpR24b5 in a bid to pin-point when the mutations evolved. The *gacS* mutation was present from the initial evolution passage, whereas the mutation in *wspF* was detected in PpR24b4 isolates from passage 7 and each subsequent passage [241], which corroborates the phenotypic observations of biofilm formation made in Chapter 3. As the *gacS* mutation evolved so early in the passage process, it may support the hypothesis that the mutation is due to improving bacterial fitness in a broth environment, rather than biofilm formation and that the mutation in *wspF* is a subsequent mutation.

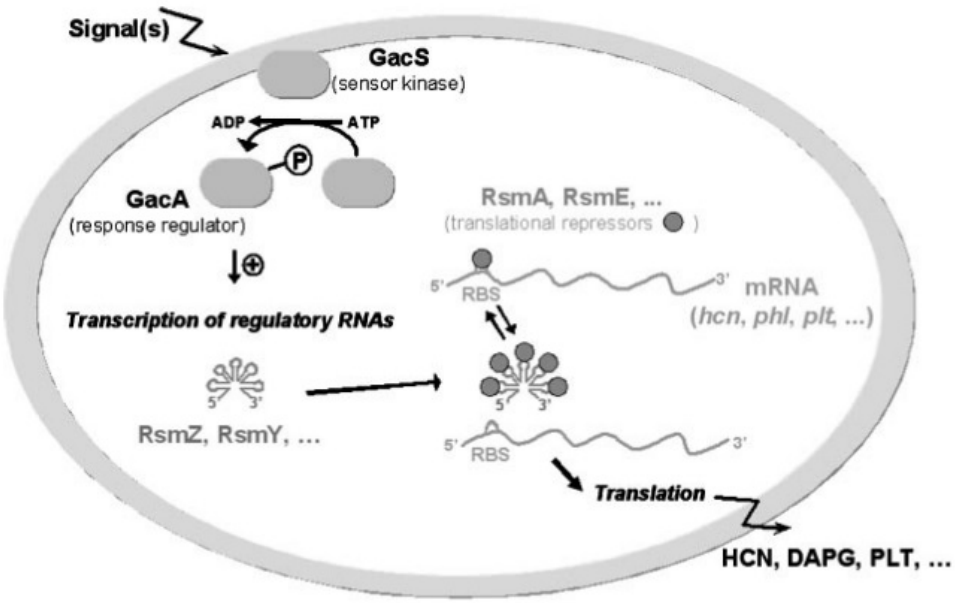


Figure 4.6: The signal transduction pathway of the GacS/GacA two-component system from [231].

4.4.3 The importance of *wspF* and *gacS* to PpR24b4 biofilm formation

The GacS/GacA pathway and c-di-GMP-regulating Wsp pathway have been identified as key mediators in the transition of bacteria from a free-floating to sessile biofilm state, therefore it could be speculated that both mutations in PpR24b4 in *gacS* and *wspF* contribute to the biofilm forming phenotype.

In light of the prior association of *wspF* and biofilm formation in the literature, it could be surmised that the SNP in *wspF* is a likely causal factor in PpR24b4's strong biofilm formation. As the mutation occurred at an active site of the N-terminal methyesterase domain, it is possible that the protein structure and function was affected and may confer knock-on effects on the regulation of *wspR*, downstream of *wspF*. This would corroborate the existing evidence that minor changes in the nucleotide sequence in *wspF* is sufficient for a bacterium to switch to a biofilm lifestyle [207]. Furthermore, the likely reduced expression of FleQ as a consequence of increased concentrations of c-di-GMP may explain the reduction in motility in isolate PpR24b4.

Similarly, there is extensive evidence in the literature associating mutations in the GacS/GacA TCS with biofilm formation and other phenotypic traits observed in Chapter 3, such as changes to siderophore production and loss of virulence factors. It could be speculated that change in microcosm pigmentation observed in the biofilm-forming isolate is regulated by the Gac system.

When the results of the evolutionary passages and trade-off analysis are viewed in context with the findings of the Capano study, it may be inferred that the initial *gacS* mutation in PpR24b4 was in response to the transition to a broth environment where phenotype switching led to the regulation of non-essential genes to improve bacterial fitness in the microcosm. Consequently, this may have resulted in changes to siderophore production, thus altering the pigmention of the broth.

As the mutation in *wspF* emerged in week 7, it is strongly indicative that the mutation in *wspF* is the causal factor in the strong biofilm phenotype that emerged in PpR24b4 in week 7. However, it is possible that the prior mutation in *gacS* was necessary for the mutation in *wspF* to occur. Alternatively, it is possible that the *wspF* mutation enhanced the actions of the *gacS* mutation by further EPS production and flagellar suppression, enabling a strong biofilm phenotype to form.

The relationship between the GacS/GacA and Wsp pathways has been discussed [219], however the direct links between the two systems remains to be elucidated. Although the presence of the *wspF* and *gacS* mutations combined with evidence from the literature suggest the SNPs detected in this study are causal factors in the PpR24b4 biofilm phenotype, further work should be conducted to substantiate these findings.

4.4.4 Suggestions for future investigations

Allele replacement or gene knock-out methodologies have previously been applied to confirm the importance of mutations in *gacS* and *wspF* in biofilm formation [207, 121, 226, 9] and such an approach could be applied in this instance to create wild-type mutants with the SNPs observed in PpR24b4, in *gacS*, *wspF*, and with both mutations which may clarify as to whether the mutations are the cause of biofilm formation. Such an approach may elucidate whether one mutation, or both, is necessary for the biofilm phenotype observed in PpR24 or indeed, whether one mutation is dependent on, or potentiates, the presence and effects of the other. Potentiation involves mutations that do not produce a distinctive phenotype but prepare the cell for future mutations that induce a phenotypic change [242, 138]. For instance, such potentiating mutations have been observed in the evolution of aerobic citrate utilising *E. coli* in the long-term evolutionary experiment [242].

The presence of only two missense mutations is in accordance with the average number of of SNPs for similar experimental evolution studies [207, 147]. However, only sequencing end-point isolates can miss a great deal of genetic variation in a population and previous studies have seen the most significant mutations that affect population fitness occur early on in evolutionary passages [198, 132]. Therefore it may be of value to delve into the 'fossil record' of the evolved isolates from each passage for a more comprehensive view on isolate evolution by sequencing each passage isolate from all lineages.

4.5 Conclusion

Hybrid assembly with variant calling analysis of the wild-type PpR24, aphid-passaged isolate PpR24a1, and biofilm-forming isolate PpR24b4 found two missense point mutation in the genes *cheB* and *barA* (homologous to *wspF* and *gacS* respectively) of PpR24b4. Mutations in both *wspF* and *gacS* have been associated with biofilm formation and reduced levels of motility and virulence in bacteria, therefore it is likely that one, or both, of these mutations are responsible for the phenotypic changes observed in PpR24b4 from the wild-type. However, further work, such as a gene knock-out mutagenesis approach, would need to be conducted to comprehensively confirm these findings.

Chapter 5

The direct and indirect effects of *Pseudomonas poae* semiochemicals on *Myzus persicae* behaviour

5.1 Introduction

The ability to repel pests from crops to minimise damage is especially advantageous in a biocontrol agent. The wild-type *P. poae* PpR24 has been observed to deter aphids when sprayed on a plant [9], however the volatiles responsible for the aphid repellent properties are unknown. This Chapter endeavours to identify the volatiles produced by the bacterium PpR24 that may be associated with insect deterrence. In addition, this Chapter will investigate whether a change in volatile emissions has occurred in the derived passaged isolates evolved in Chapter 3. Whole genome sequencing and variant calling analysis in Chapter 4 identified a loss of function in GacS, which plays a role in secondary metabolite production. Therefore, it is possible that the biofilm-forming phenotype of isolate PpR24b4 has altered volatile emissions when compared to the wild-type, which may consequently affect aphid repellency.

5.1.1 Volatile organic compounds in biocontrol

Volatile Organic Compounds (VOCs) are compounds of a low molecular mass that are produced by all organisms and play a vital role in intra and inter-

specific communication, particularly for plants, bacteria and fungi [243]. Plants are especially dependent on VOCs and phytohormones due to their sessile lifestyle. When under stress from abiotic causes or attack from herbivores and pathogens, these volatiles are an important line of defence for communicating threats to neighbouring plants as well as signalling to recruit predators capable of controlling the pest.

Plants respond to herbivory by emitting a diverse blend of compounds. These herbivore induced plant volatiles (HIPVs) mostly comprise of volatile terpenoids, aromatic compounds, such as methyl salicylate, and C_6 compounds that include alcohols, aldehydes and esters, which are commonly referred to as green leaf volatiles (GLVs)[243, 244]. The role HIPVs play in attracting predators and parasitoids is well documented. The volatiles vary depending on the herbivory the plant is subjected to. For instance, chewing pests that cause a wider extent of tissue damage, such as caterpillars, trigger different defence pathways to that of pierce-sucking phloem feeders, like aphids, which have been shown to activate defensive pathways more associated with pathogen attack [245]. Furthermore, plants can detect herbivore associated molecular patterns and alter their interactions and volatile emissions accordingly [246, 247]. This has been witnessed in the case of *Brassica nigra*, a host plant to two caterpillar species, *Pieris brassicae* and *Mamestra brassi*. *P. brassicae* is a specialist pest which has a significant effect on *B. nigra* fitness, whereas *M. brassi* is a generalist pest which leaves the plant soon after hatching. Egg laying by *Pieris brassicae* induced the plant to produce volatiles that attracted egg and larval parasitoids, which was not the case for *M. brassi*, indicating the plant has some level of herbivore recognition [248, 249].

Many natural enemies are reliant on HIPVs for prey location [250, 251]. For example, olfactory cues have been shown to be vital in Coccinellid prey location [252] and in the case of *Coccinella septempunctata*, beetles were found to positively respond to volatiles from aphid infested and recently infested plants but not uninfested plants, indicating their importance in searching and orientation behaviour [253]. Parasitoids and hyperparasitoids are also particularly sensitive to HIPVs when searching for suitable hosts, with some species even able to detect the presence of competitors in and around potential hosts [254, 255, 245, 256, 257, 258, 259].

In addition to recruiting natural enemies to a host plant, some HIPVs have been shown to have a direct effect on herbivores. In the case of aphids, some

volatile compounds produced by plants can significantly affect aphid fecundity and behaviour. As well as attracting the parasitoid *Aphidius ervi*, methyl salicylate and cis-hexen-1-ol were seen to negatively affect the aphid *Macrosiphum euphorbiae*'s attachment to the host plant and its fertility [254, 258]. Compounds may also deter aphids from colonising host plants. In a lab environment, repellencey to cis-jasmone has been observed in the grain aphid, *Sitobion avenae* [260] and methyl salicylate has been found to be repellent to the bird cherry-oat aphid, *Rhopalosiphum padi* [261, 262]. Furthermore, transgenic *Arabidopsis thaliana* plants designed to over-express a terpene synthase, consequently producing large amounts of linalool, were significantly more repellent to *M. persicae* when compared to the wild-type plants [263].

Volatiles are already used in IPM biocontrols. HIPVs and insect semiochemicals are applied in push-pull IPM strategies, by applying repellent odours to deter pests from crops and attractive compounds to lure insect pests and natural enemies [264]. For example, volatile emitting sticky-traps can be used to catch pests and methyl salicylate is an important compound for natural enemy recruitment and commercially is available as PredaLure [250]. Volatiles can also act as indicators for non-invasive monitoring of plant health for evidence of infection and pest attack [265, 266].

5.1.2 Bacteria induced plant volatiles

Bacteria use volatiles to interact with each other and their environment (Figure 5.1). Many bacteria have been shown to interact with plants and some of the best studied are the plant growth promoting rhizobacteria (PGPR). VOCs produced by these bacteria can enhance plant growth and induce systemic resistance to pathogens and pests [267]. For example, two strains of Bacillus, *Bacillus subtilis* GB03 and *Bacillus amyloliquefaciens* IN937a, produced the volatile compounds 2,3-butanediol and acetoin, which promoted growth in *Arabidopsis thaliana* and induced systemic resistance in nearby plants [268, 269]. Emitted VOCs from plant associated bacteria have also been seen to inhibit other microbial growth [270]. *Pseudomonas donghuensis* P482 VOCs inhibit the growth of several plant pathogens, including *Pseudomonas syringae* and the fungal pathogen *Rhizoctonia solani* [267].

Figure 5.1: Functions of bacteria induced plant volatiles. VOCs emitted by bacteria can enhance plant growth as well as induce systemic resistance to microbial and herbivore attack, inhibiting pathogen growth and deterring insect herbivore colonisation. Bacteria induced VOCs also act as kairomones to attract insect predators as well as prime nearby plants against impending herbivory or infection.

Paliwal [9] showed that the application of wild-type PpR24 to pepper plants had a deterrent effect on *Myzus persicae* when aphids were given a choice to colonise either a PpR24 sprayed plant or a control plant. Significantly smaller populations of aphids were found on plants treated with PpR24 when compared to control plants. Four-arm olfactometer choice chamber assays also indicated a deterrent/repellent effect. Alate *M. persicae* spent significantly less time in arms emitting volatiles extracted from plants treated with PpR24. However, this effect was not observed in arms of volatiles from PpR24 alone, implying that this was due to volatiles produced by the bacteria and plant when together.

It was hypothesised that the bacterium triggered a defence response in

the plant that led to the expression of genes in signalling pathways dependent on salicylic-acid, jasmonic acid and ethylene, which are responsible for the production of defence volatiles. This has been seen in other studies into bacteria induced systemic resistance, especially Pseudomonads and *Bacillus* species [271]. For example, when inoculated in maize roots, *Pseudomonas putida* KT2440 enhanced expression of stress-related genes used in defence, such as the production of indole, a HIPV [272, 273].

5.1.3 Aims of this Chapter

The compounds responsible for PpR24's repellent properties are unknown. Identifying the composition of volatile emissions from the bacteria may prove insightful in understanding the mechanisms of the aphid deterrent effect and how PpR24 interacts with the plant when applied as a foliar spray. However, it is possible that the point mutations in the genes *cheB* and *barA* that resulted in a strong biofilm forming isolate in Chapter 3, have also altered the volatile composition of the derived isolate, PpR24b4. PpR24b4 was able to form strong biofilms at the air-liquid interface of static microcosms but showed impaired growth on agar and in shaken environments, as well as a reduction in motility and bacterial virulence. As *barA*, or *gacS* in Pseudomonads, is associated with secondary metabolite production, it is possible that as a consequence of these mutations the volatile composition of PpR24b4 have also changed from the wild-type PpR24, at a benefit or detriment to the aphid deterrent properties.

The aims of this Chapter were to firstly identify what VOCs are produced by the wild-type PpR24 and whether the volatile composition had changed in the derived isolates PpR24a1 and PpR24b4 as a result of the experimental evolution passages. Secondly, this study aimed to identify the VOCs released when the wild-type and derived isolates were applied to a plant as a foliar spray. Finally, whether the evolved isolates PpR24a1 and PpR24b4 still retained the aphid deterrent properties of the wild type PpR24 was investigated.

5.2 Materials and methods

In this Chapter, four different protocols have been used, two to identify the volatiles produced by isolates either in a spray suspension or plant environment, and two to clarify the volatile effects on aphids.

5.2.1 Examination of bacteria and plant volatiles

Plants and bacterial spray solutions were grown, produced and applied following the methodologies described in Chapter 2.

5.2.2 Solid phase microextraction (SPME) and Gas-chromatography mass spectrometry (GC-MS) analysis of volatile compounds

Volatile compounds were extracted from the three bacterial spray treatments and 1xPBS control by Solid Phase Microextraction (SPME)(Figure 5.2). Using a 50/30 μm DVB/CAR/PDMS Stableflex fiber (Supelco, Poole, UK), 10ml of bacterial broth suspension were aliquoted into 20 ml glass SPME vials and equilibrated for 30 min at 37°C with agitation (500 rpm). The SPME fibre was then exposed to the suspension headspace for 20 min followed by desorption in the GC injection port (splitless) at 250°C. An Agilent 5975C series GC/MSD coupled to an Agilent 7890A Gas Chromatograph was used, equipped with a Zebron ZB5-MSi column (30 m x 250 μm x 1.0 μm). The oven was held at 40°C for 5 min, increased from 40°C to 220°C at a rate of 4°C/min, increased to 300°C at 8°C/min and then held at 300°C for 5 min. Helium was the carrier gas at a flow rate of 0.9ml/min. An internal standard was run before and after the samples to indicate any changes to experimental conditions. Mass spectra were recorded in electron impact mode at an ionization voltage of 70eV and source temperature of 220°C. A scan range of m/z 20-280 with a scan time of 0.69s was employed and the data were controlled and stored by the ChemStation system. Volatiles were identified by comparison of spectra and linear retention indices (based on C5-C26 alkane series) from authentic compounds.

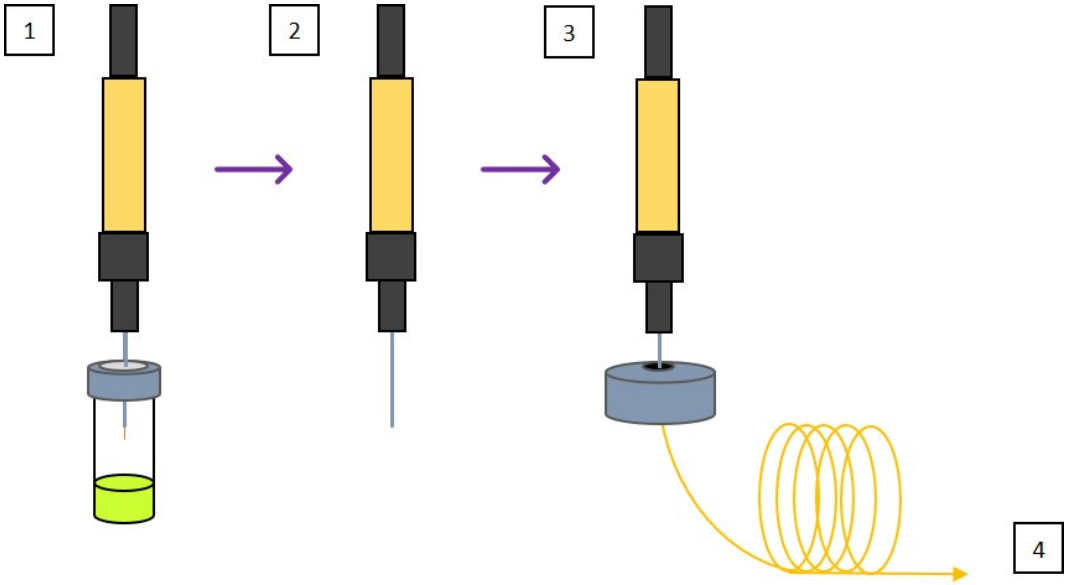


Figure 5.2: Solid Phase Microextraction and GC-MS apparatus. 1) The Stableflex fiber is exposed to the headspace at 37°C for 20 minutes. 2) The fibre is the retracted and placed into the Gas Chromatograph for desorption. 3) Desorption of volatiles occurs at 250°C onto a GC column to mass spectrometer 4). Adapted from [274].

5.2.3 Dynamic Headspace Extraction (DHE) and GC-MS analysis of volatile compounds

SPME proved unsuitable for extracting volatiles when the bacteria was applied to the plant, possibly due to the larger headspace and more complex volatile blends being produced. A more active extraction approach was required to focus the volatiles onto a trap therefore dynamic headspace extraction was used.

Four week old sweet pepper plants were sprayed with one of the four treatments, aphid-passaged PpR24a1, biofilm-passaged PpR24b4, wild-type PpR24 and control PBS as a stated in Section 2.2.6. Individual plants were left in sealed, glass flasks to equilibrate for one hour before volatile extraction occurred. The average ambient temperature was 30.5°C. Controls of soil, no-spray, and an empty jar were also recorded. Charcoal-filtered air was pulled through the glass collecting jar containing the sample plant at a flow rate of 120mL/min and volatiles were collected on a SUPELCO trap (Firgure 5.3). Volatile collection lasted one hour and after each run, the glassware was sterilised in odourless deacon and autoclaved in an oven. A 1μl of 130.6 mg/μL

Dichlorobenzene in ether standard was applied to each trap and charcoal filtered air blown over it for three minutes. Traps were then loaded onto an Analytical Thermal Desorption (ATD) machine and run for 58 minutes. Volatiles were identified as in Section 5.2.2, using the Adams library.

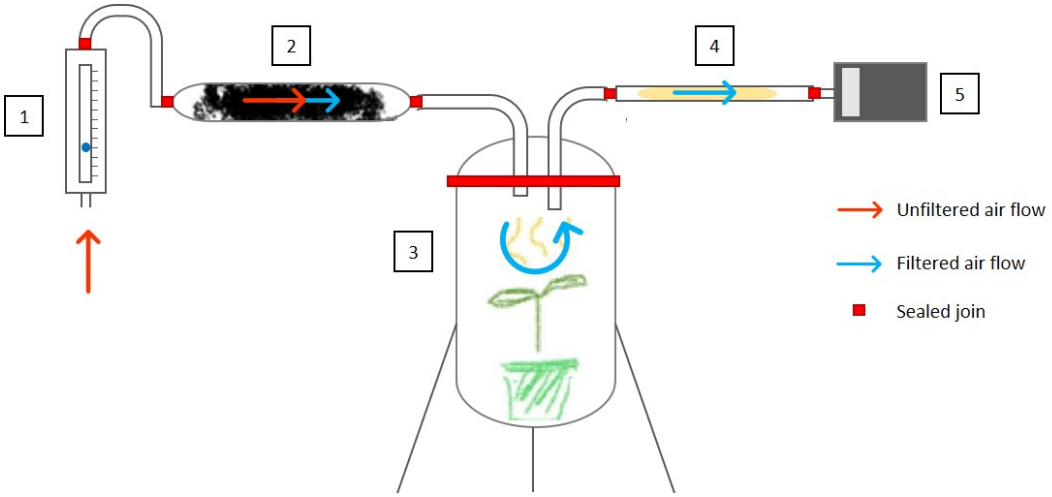


Figure 5.3: Dynamic headspace extraction apparatus. 1) Unfiltered ambient air is drawn through the apparatus by the pump (5) and flow rate recorded using a flow meter. 2) Air is filtered through a charcoal filter. 3) Filtered air is passed through the plant chamber. 4) Air from the plant chamber is purged through the SUPELCO the trap, which collects volatiles present in the sample chamber. The trap is then removed from the apparatus, 130.6mg/μL Dichlorobenzene in ether was added to the trap as a standard and traps loaded onto an ATD machine for desorption of volatiles from the trap and gas-chromatography-mass spectrometry analysis.

5.2.4 Aphid Choice Box Assays

5.2.4.1 Wild-type and passaged isolate choice box assays

Following the methodology in Section 2.2.6, plants were sprayed with their allocated treatment, either wild-type *P. poae* PpR24, aphid-passaged isolate PpR24a1, biofilm-passaged PpR24b4 or 1xPBS as a control. Two sweet pepper plants, one treated with a bacterial treatment and one control, were placed side by side in an aerated perspex box, 11.5cm x 6cm x 17.5cm, ensuring the plants were not touching. One Eppendorf containing 50 final instar aphids was placed equidistantly between the two plants. The lid was open to allow free movement to either the treated or control plant. The number of aphids on each plant was recorded after one week (Figure 5.4). Ten replicates of each treatment were

recorded and the average aphid count per plant calculated. Mann-Whitney U tests were used to assess statistical differences in preference for aphid settling behaviour.

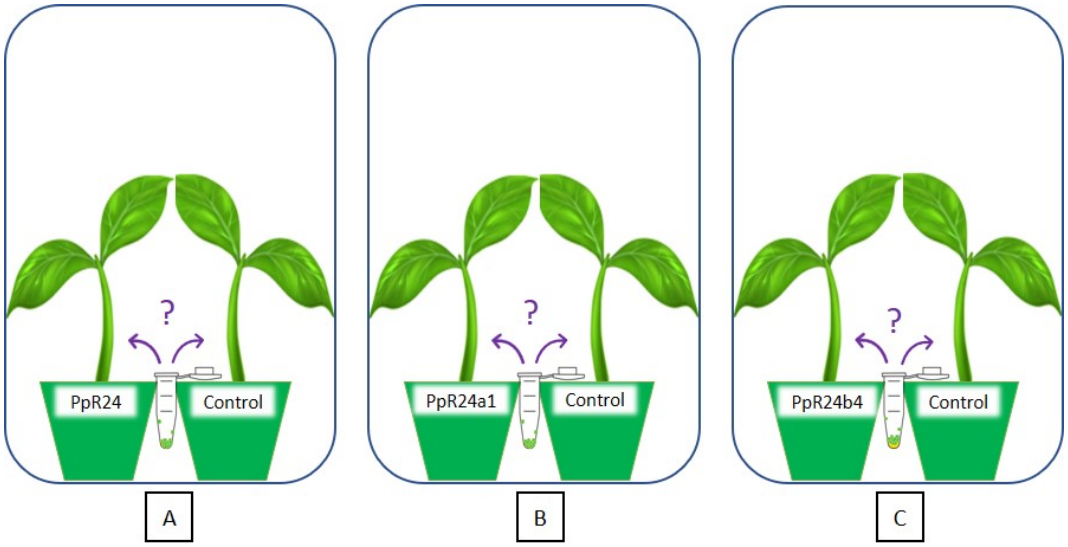


Figure 5.4: Choice box apparatus. Fifty aphids were left for one week to settle on a four week old sweet pepper plant, sprayed with either bacteria or PBS as a control. Box 'A' investigates whether aphids chose to settle on pepper plants sprayed with the wild-type PpR24 or a control PBS sprayed plant, box 'B' investigates the choice between a control PBS spray and the aphid passaged isolate PpR24a1 and box 'C' the biofilm forming isolate PpR24b4 vs a PBS control spray. Each treatment was replicated ten times.

5.2.4.2 Choice box spray post-settle assay

One untreated sweet pepper plant (Plant 1) was placed in an aerated box as described above. An open Eppendorf containing 50 adult aphids was placed at the base of the plant and left for 24 hours, allowing the aphids to settle on the plant. After 24 hours, the number of aphids settled on the Plant 1 was recorded (Day 0). Plant 1 was then sprayed following the protocol as described above with either wild-type PpR24, 1xPBS or no spray was applied and a second, untreated plant (Plant 2) was introduced to create a 'sink' for the aphids to move to if they desired (Figure 5.5). The number of aphids on each plant was recorded at 24 hours and 72 hours from the spray application. Three replicates of each treatment were conducted in triplicate.

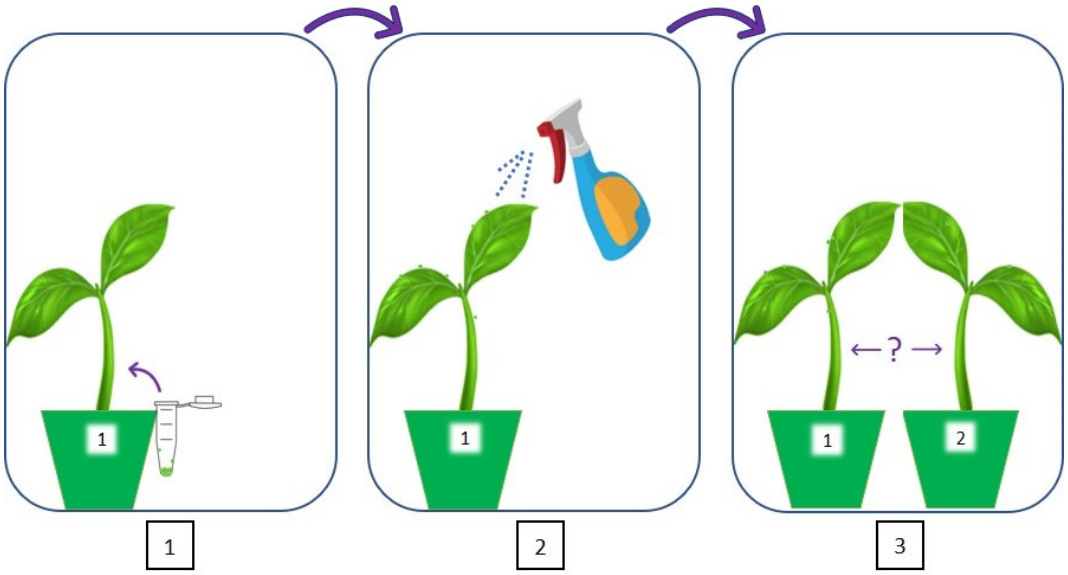


Figure 5.5: Choice box apparatus investigating the dispersal of *Myzus persicae* when sprayed after establishment on plant. For each replicate, 1) fifty aphids are left to establish on a plant for 24 hours (plant 1). 2) After 24 hours, the number of aphids on the plant are recorded and the plant is either not sprayed, sprayed with the wild-type PpR24, or sprayed with 1xPBS. 3) A second, untreated plant is introduced and aphid migration and colonisation of this plant is observed at 24 and 72 hours after plant 2's introduction.

5.3 Results

5.3.1 Solid Phase Microextraction and the identification of spray volatiles

Solid-phase microextraction and GC-MS was used to identify the volatile organic compounds emitted by the wild-type PpR24 and the two derived isolates PpR24a1 and PpR24b4 in a broth suspension, following the methodology described in Section 5.2.2. It was of particular interest to observe if any volatiles associated with insect deterrence or anti-microbial properties were present.

The extraction of volatiles from the four spray-broth suspensions, PpR24, PpR24a1, PpR24b4, and a PBS control, identified 11 putative VOCs and one unknown. The identified VOCs were: acetone, the sulphur compounds dimethyl sulphide, dimethyl disulphide, and methanethiol, three alkenes 1-undecene, 1-nonene, 1-decene and four alkanes undecane, dodecane, 2-methylpentane and tridecane (Table 5.1). The internal standard did not change more than 10% over the course of the experiment from which we can infer the experimental conditions were constant.

Variations in individual volatile levels were observed between treatments (Figure 5.6), although no significant differences were observed between the wild-type and PpR24a1 volatile levels. Isolate PpR24b4 produced lower levels of dimethyl sulphide, methanethiol, 1-decene, 1-nonene, and 1-undecene than the wild-type PpR24 and isolate PpR24a1. It is possible that the volatiles are produced in reduced quantities as a result of the mutations in *wspF* and *gacS*. The Kruskal-Wallis chi-squared test found statistical differences in compound levels between spray types found for dimethyl sulphide, dimethyl disulphide, methanethiol, acetone, 1-undecene, 1-nonene, 1-decene, and 2-methylpentane (p-values = <0.05). Dunn's post-hoc test was used to assess pair-wise differences (summarised in Table 5.1 and Figure 5.6, full pair-wise differences can be found in Appendix C). Isolate PpR24b4 had statistically higher levels of dimethyl disulphide when compared to the control PBS. In all other compounds where a significant difference was observed, pair-wise comparisons indicated the wild-type PpR24 and isolate PpR24a1 were statistically different from the control PBS.

With regards to the control PBS broth, no amounts of dimethyl sulphide,

dimethyl disulphide, methanethiol, and 1-nonene were detected,suggesting these volatiles were produced by the bacteria. Negligible amounts of 1-decene and 1-undecene were also recorded in the control broths, again suggesting these volatiles may be produced by the bacterial isolates. Higher amounts of dodecane, 2-methylpentane, tridecane, and undecane in the control microcosms may suggest that the compounds are being assimilated by the bacteria, resulting in their presence at lower levels.

Table 5.1: Compounds identified in the SPME GC-MS of volatiles from a control PBS broth, wild-type PpR24 isolate and the two derived isolates PpR24a1 and PpR24b4. Where RT - retention time/mins and p-value indicates whether a significant difference in compound levels between the three treatments and PBS control was observed.

Compound	**RT/min**	**p-value**	**Sign.**
Methanethiol	1.396	0.006187	**
Acetone	2.43	0.03613	*
Dimethyl sulphide	3.057	0.004818	**
2-Methylpentane	4.394	0.01034	**
Dimethyl disulphide	16.207	0.008992	**
1-Nonene	25.019	0.004431	**
1-Decene	29.838	0.005082	**
1-Undecene	34.188	0.005263	**
Undecane	34.556	0.9991	-
Dodecane	38.502	0.9956	-
Unknown 175	40.784	0.835	-
Tridecane	42.17	0.9991	-

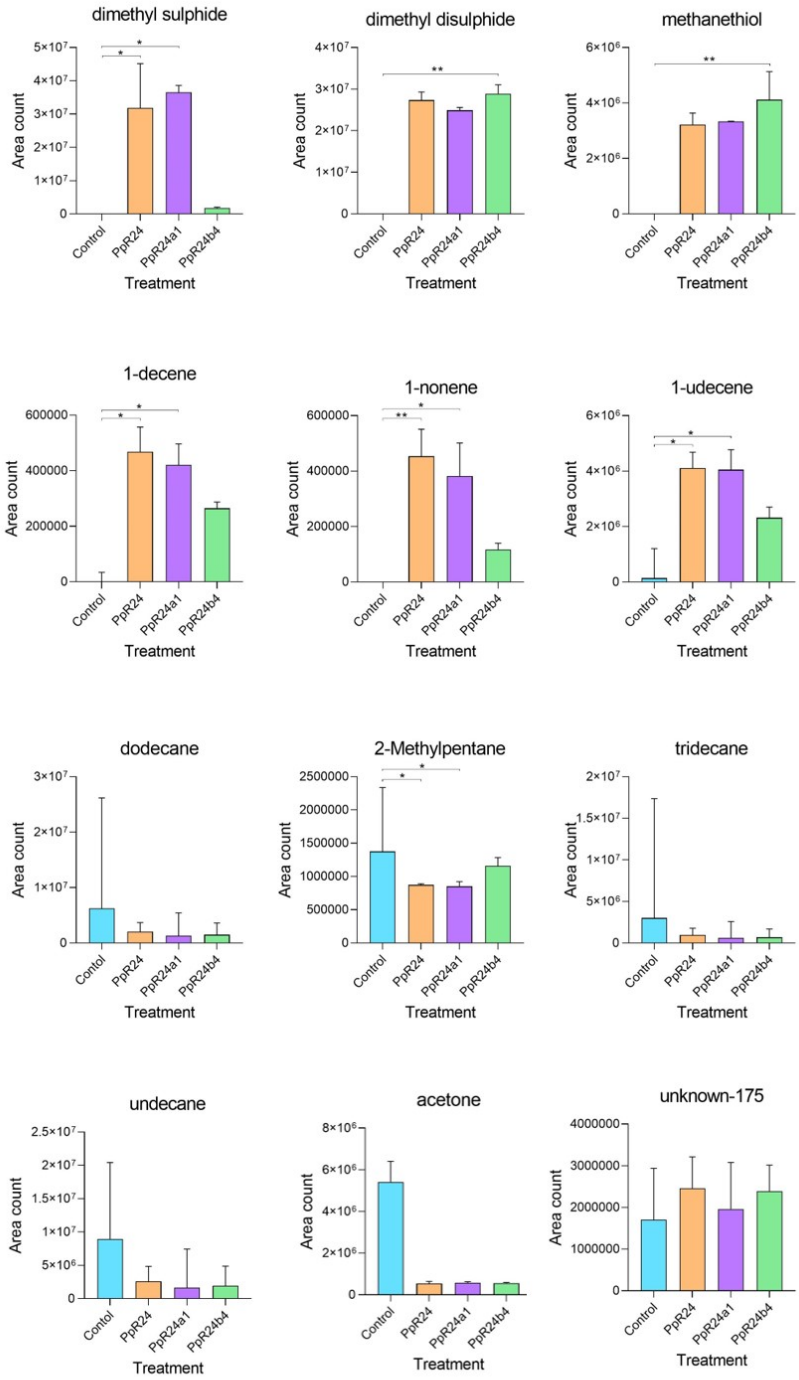


Figure 5.6: Area counts of volatile compounds detected by solid-phase microextraction and GC-MS (n=4). Median values with interquartile range bars are presented here with statistical pairwise differences from Dunn's post hoc test.

5.3.2 Dynamic Headspace Extraction (DHE) and GC-MS Analysis of Volatile Compounds

Dynamic headspace extraction with GC-MS was applied in a bid to identify volatiles responsible for the aphid deterrent effect observed in the Paliwal study that occurred when the wild-type PpR24 was sprayed on sweet pepper plants. Following the methodology laid out in Section 5.2.3, volatiles were recorded for *Capsicum annuum* plants sprayed with either the wild-type PpR24, aphid-passaged isolate PpR24a1, the biofilm-passaged isolate PpR24b4 or a PBS control spray, as well as plants with no spray treatments and the soil alone as background readings.

Seventeen volatiles were detected (summarised in Table 5.2 and Figure 5.7), none of which were present in the SPME GC-MS analysis of the bacterial-broth suspensions. Similarly, bacterial sulphur compounds present in the SPME analysis were not detected in the bacterial-plant emissions. As the volatiles detected by the DHE GC-MS mostly comprised of green-leaf volatiles, it is possible that any, more volatile, sulphur compounds that may be present are emitted at such low quantities they are masked by the green-leaf volatiles.

A great deal of variation in the levels volatiles detected was observed between treatments. By eye, no-spray and soil treatments had extremely reduced emissions of all volatiles and negligible amounts of hexenal, 2-hexenal, 3-hexen-1-ol, and 2-hexen-1-ol. Little difference was seen in the volatile spectra of the wild-type isolate PpR24, the aphid-passaged isolate PpR24a1 and the PBS spray but lower emission levels for all volatiles were observed in the biofilm isolate PpR24b4. When comparing the volatile emissions profile of each plant treatment (Figure 5.7), emissions from the soil and no-spray treatments are the background emissions. The plant response to spraying with PBS as a control showed elevated levels of 2-hexenal. The application of the bacterial isolates resulted in the detection of all the volatiles present in the soil, no-spray, and PBS control, plus the release of additional volatiles at low levels by the plant. However, significant differences were found between days which meant that it was difficult to accurately establish whether there were statistically significant differences between treatments. That being said, Kruskal-Wallis analysis found statistically significant differences in emission levels between treatments for the volatiles 3-hexenal, 2-hexenal, hexanol, 3-hexen-1-ol, 2-hexen-1-ol, 3-hexen-1-ol acetate, toluene, β-pinene, and γ-terpinene (p-values = <0.05) (Table 5.2).

Dunn's post-hoc analysis after adjustment by the Bonferroni method found significant pairwise differences in hexanol, 2-hexanal, and 2-hexen-1-ol (Table 5.3 features the significant pair-wise differences, see Appendix C for full DHE pair-wise differences).

Table 5.2: Compounds identified from DHE and GC-MS analysis searching by specific ion, where RT - retention time/mins and p-value indicates a statistically significant differences in Kruskal-Wallis chi-squared analysis of compound levels between the six treatments.

Compound	**Ion**	**RT/min**	**p-value**	**Sign.**
3-Hexenal	69	4.206	0.05379	*
2-Hexenal	69	6.17	0.01184	**
Hexanol	69	6.9	0.02144	*
Hexanal	82	4.268	0.1738	-
3-Hexen-1-ol	82	6.288	0.03205	*
2-Hexen-1-ol	82	6.7	0.02998	*
Octanol	82	12.65	0.7339	-
3-Hexen-1-ol acetate	82	12.766	0.01304	**
Toluene	93	3.39	0.05328	*
α-pinene	93	9.362	0.3847	-
β-pinene	93	11.276	0.01043	**
5-Hepten-2-one, 6-methyl	93	11.853	0.7329	-
Limonene	93	13.604	0.08649	-
3-Carene	93	14.471	0.1341	-
γ-Terpinene	93	14.876	0.03892	*
Nonanal	93	16.955	0.2394	-
Decanal	112	20.92	0.6524	-

Table 5.3: Significant pairwise differences in compound levels between plant treatments detected by DHE and GC-MS, using Dunn's post-hoc test with the Bonferroni correction.

Comparison	**Z**	**P.unadj**	**P.adj**	**Sign.**
Hexanol				
No-spray - wt	-3.12834477	0.001757938	0.02636908	*
2-Hexenal				
No-spray - wt	-2.8974706	0.003761850	0.05642775	*
Soil - wt	-3.1386575	0.001697237	0.02545856	*
2-Hexen-1-ol				
Soil - wt	-3.0365364	0.002393132	0.03589698	*

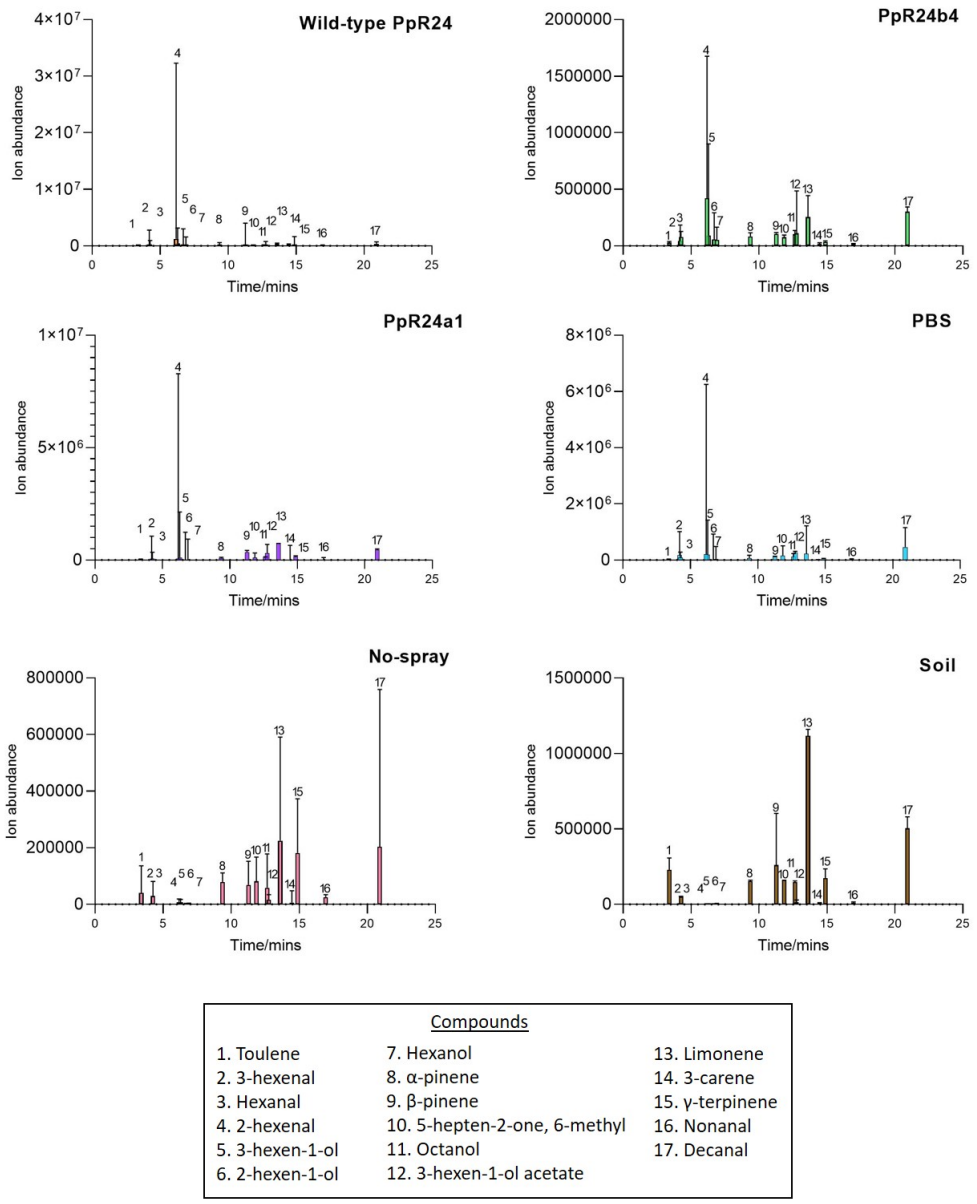


Figure 5.7: VOC emissions detected by dynamic headspace extraction with GC-MS of *C. annuum* plants sprayed with either the wild-type PpR24, aphid-passaged isolate PpR24a1, biofilm-passaged isolate PpR24b4 or a PBS spray control, with non-sprayed and soil samples visualising background emissions. Note the differing scale on the y-axis. Volatile emissions from biofilm-forming PpR24b4 sprayed plants were more reduced than plants sprayed with either the wild-type, PBS aphid-passaged isolate PpR24a1. All four spray treatments had higher VOC emissions that the soil and no-spray treatments. Median values with the interquartile bars are presented (n=4).

5.3.3 Choice box assays

5.3.3.1 Wild-type and passaged isolate choice box assays

To verify whether the evolution passages had an effect on aphid deterrence, aphids were presented with a choice to colonise either a control *C. annuum* plant treated with a PBS spray or a plant sprayed with either PpR24, PpR24a1 or PpR24b4, following the methodology in Section 5.2.4.

When presented with a choice between a plant sprayed with the wild-type PpR24 or a PBS control spray, more aphids chose to settle on the control plant than the PpR24 treated plant, which corroborated Dr Paliwal's findings of a potential deterrent effect of PpR24. However, although close to a significant p-value, no statistically significant difference was observed in aphid colonisation between the two treatments (Mann-Whitney p-value = 0.0924). Similar results were seen for the aphid-passaged isolate PpR24a1. Although by eye more aphids settled on the PBS sprayed plant, no statistical difference was observed between the two treatments (Mann-Whitney p-value = 0.4585). Likewise when presented with a choice to colonise a PBS or biofilm-isolate PpR24b4 sprayed plant, no statistical difference in aphid preference was found between the two treatments (Mann-Whitney p-value = 0.7770) (Figure 5.8).

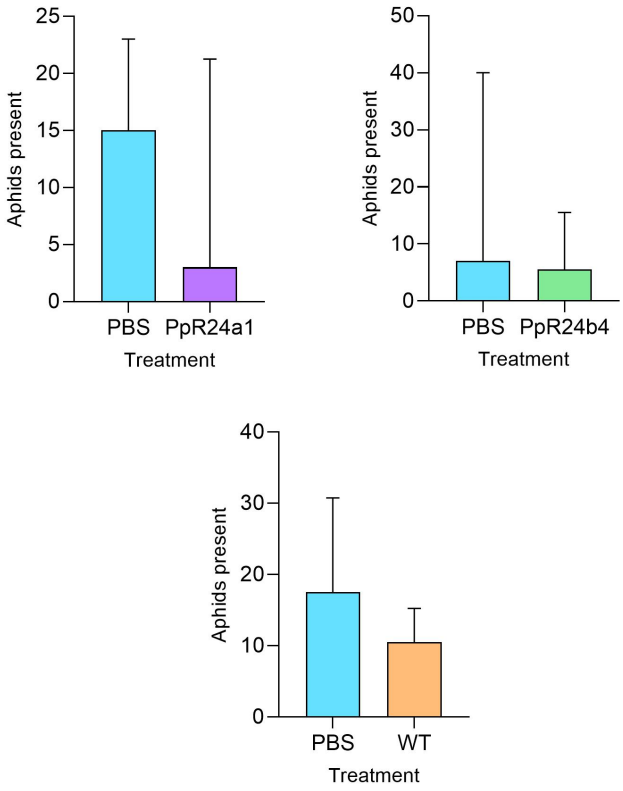


Figure 5.8: Results of the choice box assays to investigate aphid deterrence, where aphids were presented with a choice to colonise either a *C. annuum* plant sprayed with a bacterial isolate or PBS sprayed plant with served as a control. Aphid deterrent properties of the wild-type PpR24, aphid-passaged isolate PpR24a1 and biofilm isolate PpR24b4 were tested. 50 *M. persicae* were given a choice between control and treated plants (n=10). After 1 week counts of aphid presence were made. Higher numbers of aphids were recorded on the control plants for the wild-type PpR24 and aphid passaged isolate PpR24b4, although no statistical differences were found (p-values = >0.05) at the conclusion of the experiments. The median with interquartile range bars are presented.

5.3.3.2 Choice box spray post-settle assay

In order to evaluate whether the wild-type PpR24 spray was effective at dispersing aphids from an already colonised plant, assays were conducted following the methodology in Section 5.2.4. After establishing on a *C. annuum* plant for 24 hours, the original host plant (plant 1) was sprayed with either the PpR24 spray, a PBS spray, or not sprayed at all, and a second plant was introduced (plant 2) as a sink for aphids to move to, to simulate a buffer plant

zone.

All treatments saw aphids dispersing from the original host plant (plant 1) to the introduced plant (plant 2) over the 72 hour period, although less dispersal was seen for aphids on plants that were not sprayed. Statistical differences in aphid numbers were observed for dispersal from plant 1 and migration to plant 2 (Kruskal-Wallis p-values = <0.05). Dunn's post-hoc test found statistically fewer aphids were present on plant 1 for all treatments when comparing aphid numbers for 0 hour and 72 hours (WT p-value = 0.0011, PBS p-value = 0.0016, no-spray p-value = 0.0192), although no statistical difference was observed between 0 and 24 hours. Similar results were seen for migration to plant 2 for all treatments. Statistically more aphids were present on plant 2 after 72 hours (WT p-value = 0.0007, PBS p-value = 0.0007, no-spray p-value = 0.0010), but no statistical increase in aphid numbers was observed after 24 hours.

Although it appears the wild-type PpR24 and PBS spray treatments were more effective at dispersing aphids to an untreated plant, no statistical differences in aphid numbers were observed between the three treatments at 72 hours for both plant 1 and plant 2 (Kruskal-Wallis with Dunn's post-hoc test p-values = >0.05).

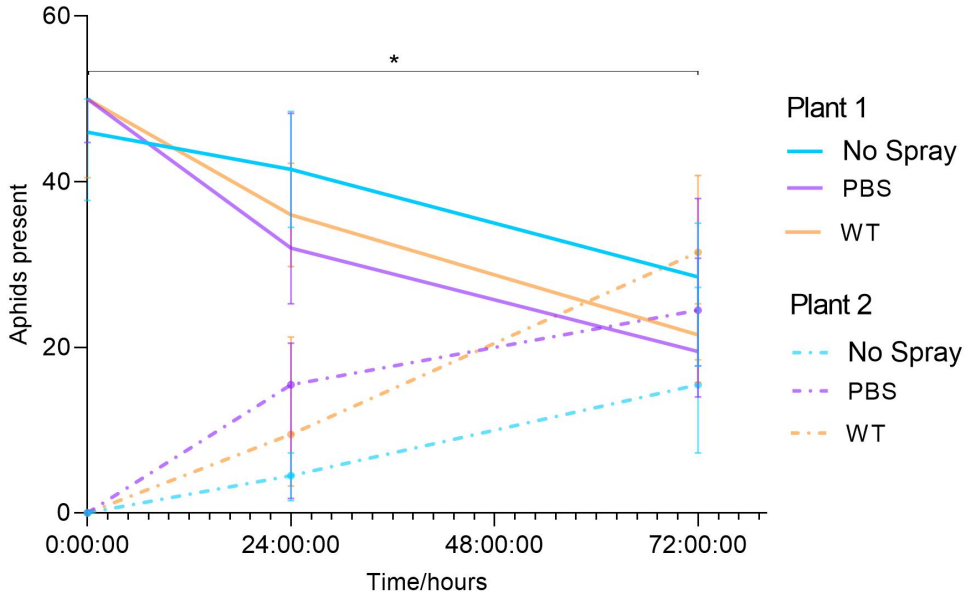


Figure 5.9: Results of spraying aphids after establishing on a host pepper plant and how it affects dispersal to an untreated plant. For each replicate, fifty aphids were allowed to establish on an un-sprayed sweet pepper plant (plant 1) for 24 hours. On day 0, plants were sprayed with either the wild-type PpR24, 1xPBS or not sprayed at all and a second, un-sprayed plant was introduced (plant 2). The number of aphids on each plant were recorded 0, 24 and 72 hours after plant 2's introduction. Aphid migration to plant 2 was observed for all three treatments. A statistical diffenence was observed in aphid numbers between 0 and 72 hours for all treatments on both plants but no statistical difference in aphid numbers was observed at each time point between treatments. Median values with interquartile ranges are presented.

5.4 Discussion

Volatile organic compounds play an important role in the interactions between bacteria, plants and insects, and there is growing interest to incorporate pest-preventing VOCs in IPM systems. Previous research found PpR24 was deterrent to aphids when sprayed on a plant [9]. This Chapter aimed to identify the VOCs emitted by PpR24 in a broth and on a plant environment in an attempt to find volatiles that may account for this deterrent action.

Experimentally evolved isolate PpR24b4 showed significant changes in phenotype from the wild-type PpR24, showing reduced motility, growth rate and aphid motility but strong biofilm formation, likely due to point mutations in *cheB* and *barA*. It is possible that changes to PpR24b4's volatile emissions also occurred, which may affect the bacterium's ability to deter aphids from a host plant as seen in the wild-type. Therefore, a comparison of the volatiles emitted by the wild-type and derived isolates, and whether changes to the deterrent properties of the isolates was also investigated.

5.4.1 Identification of volatile organic compounds

Solid-phase microextraction and GC-MS detected 11 putative volatiles from the bacteria when suspended in a spray solution. Several of the VOCs detected have been priorly associated with plant growth promotion and potential biocontrol properties [275]. As such, it is possible that the volatiles detected by SPME may contribute to PpR24's plant growth promoting properties [8]. For example, 1-nonene has been identified as a VOC that may benefit plant growth emitted by the PGPR *Pseudomonas fluorescens* SS101 [276]. Potential anti-oomycete activity has also been associated with 1-decene and dodecane in *Pseudomonas* strains [277].

The presence of dimethyl disulphide (DMDS) and 1-undecene was of particular interest. DMDS has been recognised in the volatile spectra of a variety of bacteria, including many Pseudomonads [278, 279], and is associated with anti-fungal and anti-microbial properties[280]. For instance, DMDS effectively suppresses gall growth caused by *Agrobacterium sp.* in tomato plants [106]. There is also evidence to suggest that DMDS can elicit induced systemic resistance in plants. DMDS was the dominant volatile product of *Bacillus cereus* C1L in disease control assays and when applied as a soil drench, corn and tobacco plants were protected from southern leaf blight and grey mould disease

[281]. DMDS is considered to play a defensive role when plants are damaged in *Allium* and *Brassica* species, providing protection from plant pathogens. Due to this, DMDS is under investigation as a potential biocontrol agent for fumigating soil to control plant-fungal pathogens and nematodes [106, 282]. As well as providing control against microbial attack, DMDS has been seen as an important influence of insect behaviour. Ferry *et al.* [283] found that artificially increasing the presence of DMDS around a broccoli crop significantly deterred egg laying by the cabbage fly pest, *Delia radicum*, and functioned as a kairomone, increasing the presence of the pests' natural predators.

1-Undecene is another potential anti-fungal volatile associated with rhizosphere Pseudomonads that may limit plant pathogen growth [117, 277, 279]. Exposure to 1-undecene inhibited mycelial growth of the potato blight, *Phytophthora infestans* [277]. However, the antimicrobial efficacy of 1-undecene does appear to be case dependent [106, 284]. Popova *et al.* [284] found 1-undecene to have minor antimicrobial capabilities but instead found exposure to 1-undecene to be significantly effective at limiting the development of the nematode *Caenorhabditis elegans* and caused significant mortality in *Drosophila melanogaster*. Studies into the potential application of 1-undecene as a semiochemical in attractant traps are being carried out. For example, 1-undecene is particularly attractive to the broad bean weevil, *Bruchus rufimanus*, but traps were only effective when the compound was applied with a blend of other semiochemicals [285]. Many insects rely on specific blends and ratios of VOCs for prey and host location and are extremely sensitive to slight changes in the balance of volatiles [286]. Therefore, although individual compounds may play important roles in interactions between organisms, it is vital to recognise the importance of the volatile bouquet as a whole.

Dynamic headspace extraction and GC-MS detected 17 volatiles, several of which have been associated with GLVs and HIPVs produced under stress, as well as compounds known to act as kairomones. Aldehydes and alcohols detected, such as hexanal, 2-hexenal, 3-hexen-1-ol, and 3-hexen-1-ol acetate are important GLVs in insect host plant location, such as in searching for suitable oviposition sites in stem-boring moths, *Chilo partellus* and *Busseola fusca* [287]. 2-Hexenal inhibits the growth of the fungal pathogen *Botrytis cinera*, as well as several species of bacteria, and has been thought to be emitted as part of a plant-wound response to prevent microbial infection [266]. β-Pinene has also been found to possess anti-bacterial and anti-fungal properties

[280]. α-pinene and limonene have previously been associated with volatiles induced by aphid herbivory that act as kairomones to attract the parasitoid *A. ervi* [258].

Although SPME analysis detected significant differences in compound levels between the bacterial isolates and the PBS control, no statistical differences were observed in pairwise comparisons between isolates. As there were no significant phenotypic or genetic changes between PpR24 and PpR24a1, this was expected. However, although not statistically significant, lower levels of 1-decene, 1-nonene and DMDS were seen in the biofilm isolate PpR24b4. Similarly, DHE and GC-MS analysis showed reduced levels of green leaf volatiles produce by PpR24b4 compared to the wild-type PpR24 but these differences were not statistically important. It is likely that the differences in volatile emissions in the evolved isolate PpR24b4 are due to the mutations in *wspF* and *gacS* genes identified in Chapter 4.

5.4.2 The role of GacS and WspF in volatile emissions

In Pseudomonads, the GacS/GacA regulatory system has been associated with volatile organic compound and secondary metabolite production [288]. *Pseudomonas fluorescens* SBW25 GacS mutants showed reduced levels of volatile compound production, including 1-undecene and dimethyl sulphide, indicating volatile products are at least in part regulated by the GacS sensor kinase [224]. Similarly in *Pseudomonas chlororaphis* PA23, *gacS* mutants had reduced production of anti-microbial compounds and volatile hydrogen cyanide [289]. GacS was also shown to regulate the production of 2R,3R-butanediol in *Pseudomonas chlororaphis* O6, which confers resistance to *Erwinia carotovora* subsp. *carotovora* SCC1 in tobacco [290]. However, volatile production is not exclusive to the GacS/GacA regulatory system. In *Pseudomonas donghuensis* P482, dimethyl sulphide was found to be regulated by the GacS/GacA system whereas dimethyl disulphide was not [267]. This may explain why dimethyl sulphide was found at lower levels in the PpR24b4 isolate compared to the wild-type.

The mutation in *wspF* may also contribute to the change in volatile emissions due to its role in phenotype switching between sessile biofilm and motile state. As explored in Chapter 4, simple mutations in *wspF* result in the constitutive production of WspR, which contains a conserved C-terminal GGDEF

domain associated with the production of the intracellular signalling molecule, cyclic diguanylate (c-di-GMP) [213]. High levels of c-di-GMP result in increased cell aggregation, surface attachment, and consequently biofilm formation. As well as mediating the regulation of adhesive substances and extracellular polysaccharides, c-di-GMP can repress virulence gene expression and other factors associated with a motile phenotype [291, 292]. This may account for the reduced levels of volatiles associated with anti-microbial properties detected.

5.4.3 Limitations of volatile extraction

None of the volatiles detected in the SPME analysis of the spray treatments were present in the DHE of treated plants. It is possible that the sulphur compounds produced by the bacteria are in such low quantities in comparison to the green leaf volatiles emitted by the plant that they are undetected by the set-up. Large amounts of noise in the volatile spectra may also mask the presence of small, highly volatile bacterial compounds with a low retention time. Although significant differences between bacterial treatments and the PBS control were seen in SPME analysis, no statistical differences were detected in the DHE GC-MS of pepper plants sprayed with bacteria or PBS control spray. It is possible that the act of spraying alone, regardless of the treatment, is enough to trigger the emission of the GLVs.

Plants have been seen to emit GLVs in response to physical disturbance. Most plants emit low-levels of GLVs in an undisturbed state, which may account for their presence in the no-spray control plants. However, plants are extremely sensitive to abiotic stresses such as physical disturbance [243]. Therefore it is more likely the volatiles are emitted as a result of moving the sample plants into the experimental conditions, which should be reduced as much as possible in future replications of this method.

5.4.4 Isolate effects on aphid host plant selection

When presented with a choice to settle on either a control or a wild-type spray plant, statistically more insects settled on the control plant. This corroborated the previous study, where in an olfactometer setting the combined extracted volatiles from plants sprayed with PpR24 deterred winged *M. persicae* [9]. No significant difference was seen in aphid host plant choice when aphids

were presented with a control PBS sprayed plant and plants either sprayed with PpR24b4 or PpR24a1. It is possible that the loss of deterrency is due to differences in the levels of compounds emitted by PpR24 and PpR24b4 that were detected by SPME and DHE, such as a reduction in dimethyl sulphide.

However, no significant genetic, phenotypic or volatile difference was seen between the wild-type PpR24 and aphid-passaged PpR24a1 but there was no statistical difference between aphids colonising control or PpR24a1 sprayed plants, implying a loss of deterrency. It may be that the method of volatile detection is not sensitive enough to discern subtle differences in the emissions and that volatiles are lost in the noise of the spectra. Olfactometer assays may be used to confirm whether it is solely due to volatiles, coupled with electro-antennography to identify specific compounds or blends that aphids respond to. On the other hand, visual factors may also influence aphid host-plant choice. It is possible that aphids are able to detect the presence of the bacteria as they probe for a suitable feeding site and are repelled by the bacteria's presence [293, 294].

There is evidence to suggest that the act of spraying a plant is enough to induce the emission of GLVs to instigate the movement of *Myzus persicae* off a host plant. Although there are serious issues with the experiment preventing definitive conclusions, DHE-GC-MS analysis found applying a PBS spray caused similar blends of VOCs to be emitted from the plants to that of the wild-type. Indeed, the soil and plants with no-spray treatment produced significantly less GLVs than all the spray treatments, including the PBS spray. In addition, when plants with established aphid colonies were sprayed with either a control PBS or wild-type treatment, both treatments resulted in the dispersal of aphids to a new, un-sprayed plant, with more aphids settled on the fresh plant that the original after three days. It may be that the movement of aphids is due to the disturbance caused by the droplets, rather than the volatiles induced in the plant by the treatments. For instance, when disturbed on a plant some aphid species drop off the host plant as an anti-threat response [295]. It may be possible that a similar behaviour is being exhibited here.

5.5 Conclusion

This Chapter identified key volatile components from the headspace of the wild-type PpR24 and its derived isolates in broth and when applied on a

plant. The biofilm forming isolate PpR24b4 showed significant differences in compound levels, particularly lower levels of dimethyl sulphide and 1-undecene. The difference in volatile composition may be attributed to the mutations detected in the *cheB* and *barA* genes identified in Chapter 4, which have both been associated with secondary metabolite production.

Many of the volatiles identified have been previously associated with induced plant defence strategies, which may explain the deterrent effect the wild-type bacteria has on aphids. However, no significant difference was observed between the volatile emissions of plants sprayed with PBS and plants sprayed with PpR24 but this was most likely due to high variability in the data. More replicates and reduced plant disturbance may help reduce this 'noise'.

In choice assays between PBS and bacteria sprayed plants, a deterrent effect was observed for the wild-type PpR24 but not for isolates PpR24a1 and PpR24b4, implying that deterrency was lost during the evolution process. However, spraying a plant with an established aphid colony with PBS was effective at inducing movement to a new plant, as was PpR24.

Chapter 6

Pseudomonas poae's effects on non-target natural enemies

6.1 Introduction

Beneficial insects are naturally occurring species that are found in agricultural habitats. These species provide farmers with many important ecosystem services, such as pollination and pest control. There are two main ways to encourage the provision of these services from beneficial insects: firstly supporting naturally occurring populations by providing suitable on-farm resources to enhance their numbers, and secondly to release additional numbers of insects from commercially reared sources. Natural enemies are insects that naturally target a pest species and are often a key component in IPM systems where they are introduced or encouraged into crop systems as part of augmentative and conservation biocontrol programs. Amid concerns over the frequent and intensive application of chemical pesticides on consumer, grower, and environmental health, there is a global shift towards alternative strategies for pest control in agriculture away from the use of chemical pesticides. As well as providing growers with effective pest control at minimal risk to worker and consumer health, insect natural enemies remain effective against pesticide resistant pests.

When used alone, insect natural enemies do not have a 100% success rate therefore it is common practice for growers to use such beneficials alongside other methods of control, for instance sticky traps, volatile traps, and compatible chemical-based or biological pesticides that cause less than 20% natural

enemy mortality [296]. Studies have shown that chemical pesticides can be used alongside pest natural enemies, however, findings vary greatly between studies with several demonstrating that chemical pesticide products have adverse non-target effects on beneficials, often resulting in the extinction of the beneficial in the crop [297, 298, 32, 34, 299, 300, 40, 41, 43]. These non-target effects may manifest in different ways. There are obvious lethal effects but also sub-lethal effects on fecundity, offspring development rates, and effects on natural enemy behaviour that can influence how an insect interacts with the crop/species pest [301, 302]. Therefore, when forming holistic crop management schemes it is vital to ascertain whether a microbial biopesticide is suitable for use alongside beneficial insects.

In previous investigations, *Pseudomonas poae* PpR24 has been shown to have a high specificity to aphid species, proving non-harmful to non-target insects in a series of laboratory exposure tests (Table 6.1).

Table 6.1: Non-target effects of PpR24 investigated in the Hamilton study [8]. None of the insects were significantly affected by PpR24 in the routes of exposure tested.

Species	**Order**	**Exposure to PpR24**
Galleria mellonella	Lepidoptera	Larvae, injected, inoculated food
Oryzaephilus surinamensis	Coleoptera	Larvae, inoculated food and diet
Sitophilus oryzae	Coleoptera	Larvae, inoculated food and diet
Cryptolestes capillulus	Coleoptera	Larvae, inoculated food and diet
Apis mellifera	Hymenoptera	Larvae, inoculated diet

This chapter takes this investigation further by focussing on the potential direct, lethal effects of *Pseudomonas poae* PpR24 on three commercially available beneficial insects by simulating likely routes of exposure the insects may encounter in the glasshouse crop environment. *Orius laevigatus*, *Macrolophus pygmaeus* and *Aphidius colemani* are three natural enemies produced for aphid control that are commonly applied in glasshouse sweet pepper systems [303, 304].

6.1.1 *Aphidius colemani*

Several species of parasitic wasps have evolved with a high specificity to aphids, for instance *Aphidius ervi*, *Aphidius colemani*, *Aphidius metricarae* and

Diaretilla rapae [264, 24]. *Aphidius colemani* Viereck (Hymenoptera: Braconidae) [305] have been used in biocontrol programs since the early 1970s [305]. *A. colemani*'s popularity in glasshouse environments is partly due to their wide host range, as they are able to parasitise 41 aphid species [305, 306]. Similar to other members of the Braconidae, *A. colemani* is an endoparasite koinobiont, where the wasp spends all of its developmental stages inside the aphid, which continues to feed even when parasitised [307]. A female wasp lays a single egg inside an aphid which then develops for three larval instars. During the last instar, the developing parasitoid kills its host and spins a silk cocoon inside the deceased aphid. Before pupation, the aphid cuticle hardens and dries, ballooning slightly into husk called a mummy. Once pupation is complete, the wasps cut a circular hole in the mummy to emerge [307, 308] (Figure 6.1).

Aphidius colemani can be highly effective at controlling aphids when applied as a preventative measure or as augmentative control once an aphid infestation is present in the crop. *A. colemani*'s high specificity to their aphid host has resulted in them being able to detect and respond aphid signals, such as being attracted to aphid sex pheromones [309, 310]. For instance, when applied as a preventative measure against *M. persicae*, as few as 0.15 individuals are required per m^2, although this increases to 1.5 per m^2 if an infestation has established [307]. Often in preventative strategies, parasitoids are reared in banker plant systems alongside the main crop. For example, in sweet pepper glasshouses, wasps may be reared on wheat seedlings deliberately infested with *Rhopalosiphum padi*, an aphid that does not feed on pepper crops but that is still a viable host for *A. colemani*. Using such techniques allows the wasps to be continuously present in the system and able to quickly control the target aphid pest species on sweet pepper plants [307, 305].

However, *A. colemani* are not able to parasitise all aphid species. For instance, although capable of stinging the potato aphid, *Macrosiphum euphorbiae*, *A. colemani* are unable to successfully use *M. euphorbiae* as hosts as the wasp's eggs cannot develop inside the aphid [305]. Due to this aphid specificity, parasitoids are considered most successful when used alongside other predator species as part of a management strategy rather than being solely responsible for aphid control [24, 71]. However, developing wasps can suffer from intra-guild predation from other predators in an IPM system, such as ladybirds, and can be susceptible to hyperparasitisism [311].

Figure 6.1: *Aphidius colemani*. Wasps oviposit into live aphids. Larvae develop inside the aphids, eventually killing the aphid host forming a mummy. Wasps cut a circular hole in the hardened cuticle to escape once reaching maturity (Images taken from [312, 313, 314])

6.1.2 *Orius laevigatus*

The flower bug, *Orius laevigatus* Fieber (Hemiptera: Anthocoridae) [315] is a generalist insect predator originating from the Mediterranean that is commonly used to control thrips, aphids, mites and whitefly [297, 51, 316, 317] (Figure 6.2). Often applied in glasshouse and outdoor crops [297], *Orius* species are especially effective due to their ability to hunt prey in concealed locations, such as in flower buds [296]. *Orius* species are also able to survive on plant pollen, enabling them to persist in a crop even when insect prey are at low population density [315, 317], making them an ideal species for preventative control. In pollen producing crops, only 0.5-1 individual per m^2 is required for effective preventative control, although post-outbreak, 5-10 individuals per m^2 are necessary for sufficient pest suppression [318].

Females lay eggs inside the plant tissue, which hatch after approximately 5 days. The nymphs then undergo five development instars before reaching adulthood after roughly three weeks, dependent on temperature. Similar to aphids, *Orius* species possess the elongated, piercing mouth parts characteristic of Hemipterans the insects use to pierce insect prey and plant tissue to feed [319, 316].

Although a popular and useful biocontrol agent, *Orius* species have proven to be susceptible to intraguild predation when used alongside other predators, such as *M. pygmaeus* [304], and some insecticides applied in IPM systems [51, 320, 297].

Figure 6.2: *Orius laevigatus*. *O. laevigatus* predate on pests even during instar development (Images taken from [321, 322, 323])

6.1.3 *Macrolophus pygmaeus*

The mirid bug *Macrolophus pygmaeus* Rambur (Hemiptera: Miridae) is a polyphagous, predatory insect native to the Mediterranean that is commonly employed against whitefly, thrips and aphids [302, 324, 325] (Figure 6.3). *Macrolophus pygmaeus* have a similar life-cycle to that of *O. laevigatus* and also use their piercing, sucking mouthparts to feed on insect and plant matter [324].

However, at low prey densities or when *M. pygmaeus* populations get too large, *M. pygmaeus* can cause crop damage. This may take the form of aesthetic damage to fruit but also, more seriously, flower damage which may lead to fruit abortion and lower crop yields [326]. Due to this, the recommended application dosage of *Macrolophus* individuals varies with crop, target pest, and intensity of infestation.

Figure 6.3: *Macrolophus pygmaeus*. *M. pygmaeus* uses its rostrum to stab prey and feed. When not in use, the straw-like apparatus is folded under the ventral side of the insect (Images obtained from [327] and [328])

6.1.4 Aims of this Chapter

Identifying potential lethal effects of PpR24 may prove insightful in assessing the suitability of using the bacterium alongside the three natural enemies mentioned above. This Chapter applied lab-based protocols to simulate three likely routes of biopesticide exposure that natural enemies may encounter.

Methods frequently used to assess the effects of pesticides on non-target insects were used. Firstly, topical application of PpR24 droplets directly onto the insect cuticle simulated direct exposure to the bacterial foliar spray [329, 330, 43]. Secondly, a residuals assay was conducted where natural enemies were exposed to spray residues on excised leaves [329, 320]. Finally, an oral exposure assay examined whether feeding on PpR24-fed *Myzus persicae* had a lethal effect on *O. laevigatus* and *M. pygmaeus*.

Only the wild-type PpR24 was used in this Chapter as in consideration of the results obtained in Chapters 3 and 5, it remained the most likely biopesticide candidate when compared to its derived isolates.

6.2 Methods

Orius laevigatus, *Aphidius colemani* and *Macrolophus pygmaeus* were produced by Biobest and supplied by Agralan growers. Insects were kept in a controlled environment room at 25°C on a long-day light cycle (16/8 hours). Assays were conducted under the same conditions. Four-week old sweet pepper plants and adult predators, parasitoids, and aphids were used. For each assay, mortality assessment was conducted at 72 hours and insects were considered dead if they failed to move after a light prod with a paint brush. For topical and residual assays, the insects were supplemented with food to ensure they did not starve to death. *Orius laevigatus* and *M. pygmaeus* were supplemented with Nutrimac *Ephestia kuehniella* eggs produced by Biobest. *Aphidius colemani* were nourished with 30% honey water.

6.2.1 Topical assay

Insects were chilled at 4°C for 3-5 minutes. This was sufficient time to immobilise them to allow droplet application without causing lasting harm. A 10μl micropipette was used to administrate 1μl of wild-type PpR24 at 10^7 concentration (in 1xPBS) onto the dorsal side of the insects. 'Non-spray' control insects were not treated and PBS controls were treated with 1μl of 1xPBS as a control for the droplet procedure. Ten insects were used per replicate, with each treatment replicated ten times (100 insects per treatment). Supplementary food (as stated above) was provided for the duration of the assay.

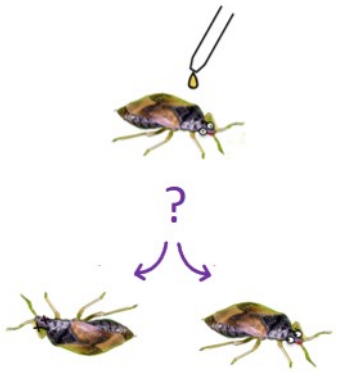


Figure 6.4: Topical assay. A 1μl droplet of PpR24 is administered onto the dorsal side of the insects. After 72 hours, observations are made as to whether the insects are alive or dead.

6.2.2 *Aphidius colemani* mummy emergence

Ten mummified aphids of *A. colemani*, were placed in a Petri dish. As in Section 6.2.1, 1μl of wild-type PpR24 at 10^7 concentration was pipetted onto the mummies. 'Non-spray' control mummies were not treated with a droplet and PBS controls were treated with 1μl of 1 x PBS as a control for the droplet procedure. Wasp emergence was recorded over four days.

6.2.3 Residual spray assay

A spray suspension of the wild-type PpR24 was made up following the method in Chapter 2, Section 2.6, and 1ml was applied to the abaxial and adaxial surface of an excised *Capsicum annuum* leaf. A 1xPBS spray was applied to control leaves. Leaves were left to dry in a laminar flow cabinet until completely dry, then placed in aerated boxes. Five predators or parasitoids were placed in each box and left for 72 hours, after which mortality was observed. Insects were provided with supplementary food for the duration of the experiment (as stated above).

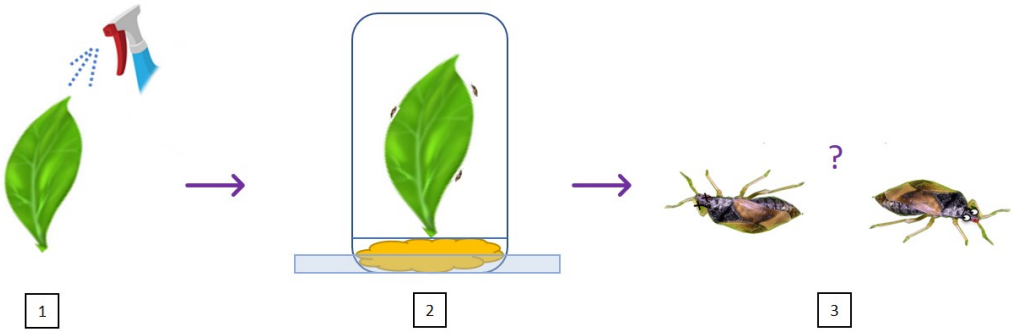


Figure 6.5: Residuals assay. Foliar spray was applied to the abaxial and adaxial sides of excised sweet pepper leaves and left to dry. Insects were left to walk on the exposed leaves for 72 hours, after which time mortality observations were recorded.

6.2.4 Oral assay

Aphids were allowed to feed on artificial diet sachets containing PBS control or wild-type PpR24 for 24 hours, as described in Chapter 2, Section 2.5. Ten final instar aphids were then placed in a Petri dish with one predator insect and the dish sealed with parafilm. The predators were left to feed on the aphids for 72 hours and their mortality observed.

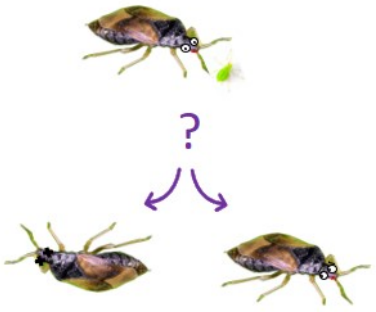


Figure 6.6: Oral assay. Aphids were fed on artificial diet inoculated with PpR24 for 24 hours, after which time they were exposed to the predator insects (either *M. pygmaeus* or *O. laevigatus*). The predators were left to feed on the aphid and after 24 hours mortality was recorded.

6.2.5 Statistical analysis

All statistical tests were conducted in R, version 3.6.1. The Kruskal-Wallis rank sum test and Dunn post-hoc test was used to analyse the results of the topical and mummy emergence assays, with p-values adjusted using the Bonferroni method. Residual spray assays were analysed using the Mann-Whitney test. The Fisher's exact test was used to analyse the oral assay.

6.3 Results

6.3.1 Topical assay

To assess whether direct exposure to PpR24 had a lethal effect on *O. laevigatus*, *A. colemani* and *M. pygmaeus*, insects were treated with 1μl droplets of PpR24 foliar spray suspension as described in Section 6.2.1.

After 72 hours, for all three insect species the highest mortality was observed for wild-type PpR24 treated insects. No statistical differences were observed between the PBS and no-droplet treatments for any of the natural enemy species tested (Figure 6.7).

When *O. laevigatus* insects were exposed to the three droplet treatments, significant differences in insect mortality between the three treatments were detected (Kruskal-Wallis p-value = <0.05). Statistically higher mortality was observed in insects treated with PpR24 droplets when compared to insects treated with PBS droplets (Dunn's post-hoc pairwise comparison p-value = 0.0170) or not-treated (Dunn's post-hoc pairwise p-value = 0.0004).

Similarly in the case of *A. colemani*, statistical differences in aphid mortality were also observed between the three droplet treatments (Kruskal-Wallis p-value = <0.05). Post-hoc pairwise analysis found statistically higher mortality in *A. colemani* insects treated with PpR24 when compared to no-treatment insects (Dunn's post hoc pairwise p-value = 0.0439).

However, no statistically significant differences in insect mortality were observed between the three treatments for *M. pygmaeus* insects (Kruskal-Wallis p-value = >0.05).

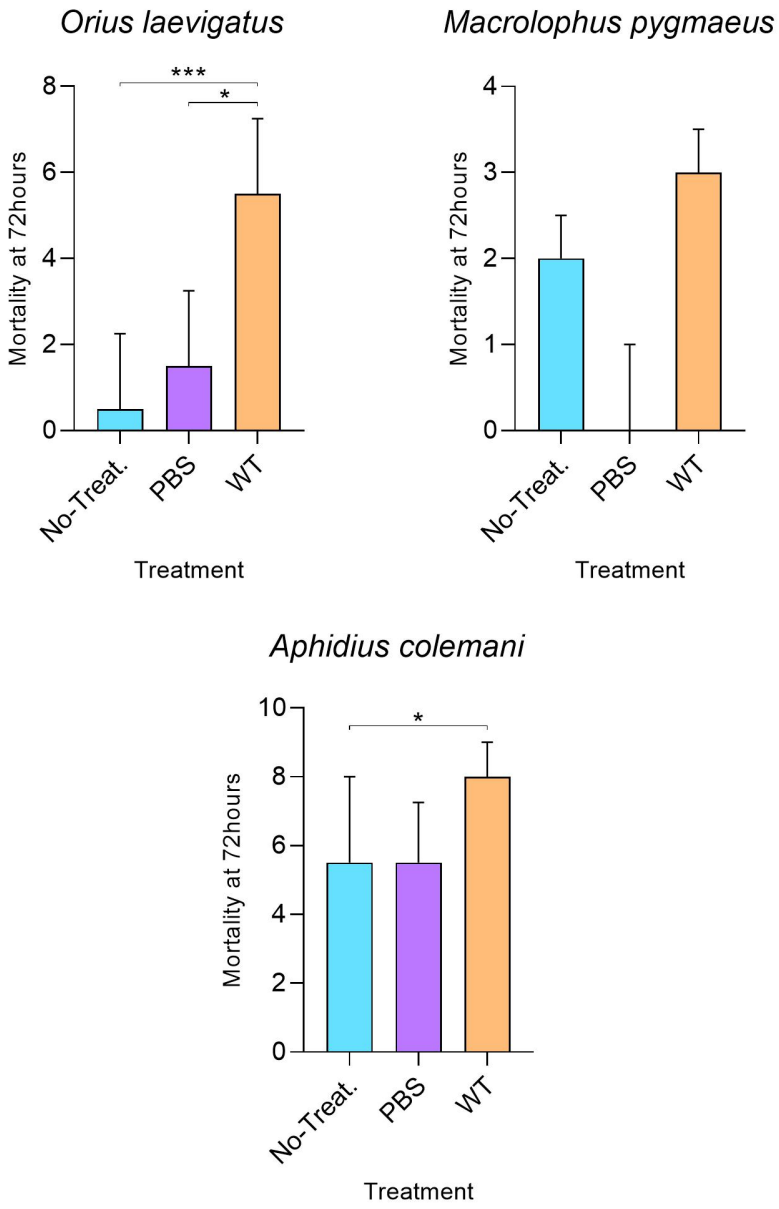


Figure 6.7: Topical assay results for *O. laevigatus*, *M. pygmaeus* and *A. colemani*. Insect mortality was recorded at 72 hours. Median values and interquartile range are present with significant Dunn's post hoc pairwise differences, n=10. Note the differing y-axis scales.

6.3.2 *Aphidius colemani* emergence

To investigate whether PpR24 exposure affected *A. colemani* mummy emergence, topical application of PpR24-suspension droplets were applied to mummified aphids following the methodology in Section 6.2.2 to simulate foliar-spray contact.

Over the course of 72 hours, mummy emergence was recorded in 24 hour intervals. In the first 48 hours of the assay, no statistical differences were observed in wasp emergence between the three treatments. At 72 hours, statistical differences in mummy emergence were observed (Kruskal-Wallis p-value = <0.05), with post-hoc verifying significantly fewer wasps emerged when mummies were treated with PBS when compared to untreated mummies (Dunn's post-hoc test p-value = 0.0044). However, no statistical differences were observed between PpR24 and control treatments which may indicate no overall treatment effect (Figure 6.8). (Full post-hoc pairwise comparisons can be found in Appendix D).

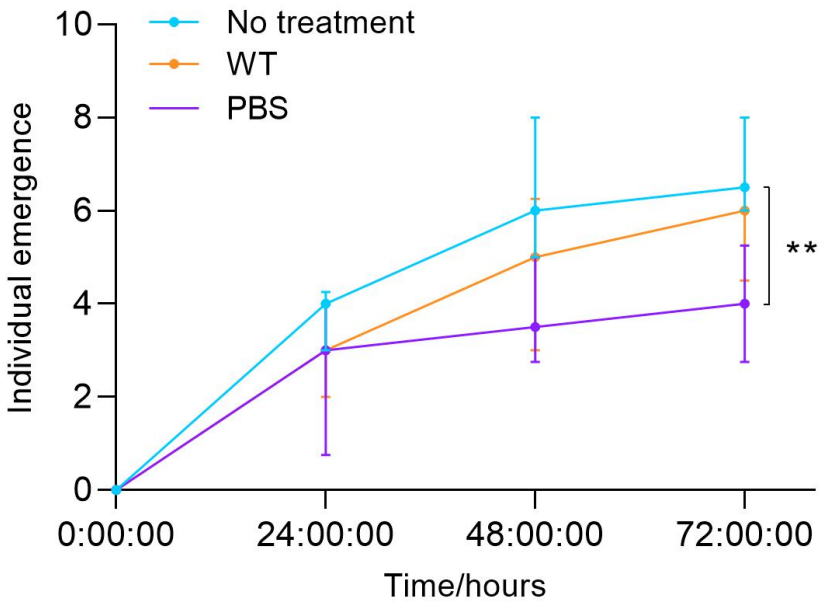


Figure 6.8: *Aphidius colemani* emergence from mummies over 72 hours, where 'control' treatment is no droplet, 'PBS' a control PBS droplet and 'WT' the wild-type PpR24. Median values with interquartlie range are presented with significant Dunn's post-hoc pair-wise differences.

6.3.3 Residual spray assay

To observe the effects of PpR24 spray residues on *O. laevigatus*, *A. colemani* and *M. pygmaeus*, excised *Capsicum annuum* leaves were sprayed following the protocol in Chapter 2, Section 2.6, and left to dry before exposing the natural enemies to the leaves (Section 6.2.3).

After 72 hours of exposure to the sprayed leaves, mortality was observed in all insect species (Figure 6.9). Although higher mortality was observed for insects exposed to PpR24 sprayed leaves, no statistical differences were observed in insect mortality between the PBS and PpR24 spray treatments for *O. laevigatus* and *M. pygmaeus* insects (Mann-Whitney test p-value = >0.9999).

On the other hand, a statistically significant difference was found between the control and PpR24 treatments for *A. colemani* insect (Mann-Whitney test p-value = 0.04), implying a potential susceptibility to residue exposure.

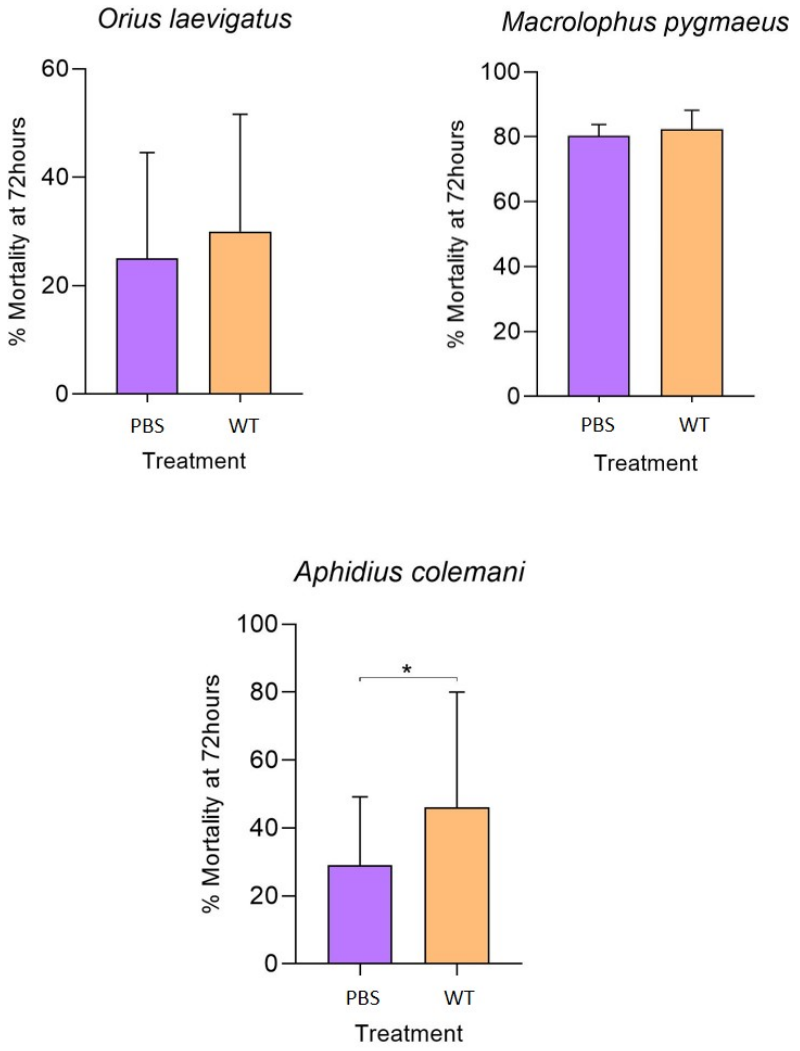


Figure 6.9: Natural enemy exposure to PpR24 spray residues. After exposure to excised pepper leaves treated with either a PBS control or wild-type PpR24 foliar spray suspension for 72 hour, no statistical differences were seen between treatments for *O. laevigatus* and *M. pygmaeus* insects. A statistical difference in insect mortality was observed for *A. colemani* (Mann-Whitney test p-value = 0.04). Medians and interquartile ranges are presented.

6.3.4 Oral assay

As pierce-sucking predators, *O. laevigatus* and *M. pygmaeus* have the potential to ingest PpR24 when feeding on aphid prey. To investigate whether PpR24 could have a lethal affect in such a tri-trophic interaction, *O. laevigatus* and *M. pygmaeus* were left to feed on aphids fed artificial Mittler diet inoculated with PpR24 (following the methodology in Section 6.2.4).

No statistical difference in *M. pygmaeus* survival was observed between insects left to feed on aphids fed PpR24 or control diet (Fisher's exact test p-value = >0.9999)(Table 6.2). It may be inferred that the ingestion of PpR24 fed aphids did not significantly affect *M. pygmaeus* mortality.

However, statistically higher mortality was observed for *O. laevigatus* insects left to feed on PpR24-fed aphids (Fisher's exact test p-value = 0.0325)(Table 6.3).

Table 6.2: *Macrolophus pygmaeus* ingestion of *M. persicae* fed on PpR24. No statistical difference was observed in mortality between *M. pygmaeus* insects predating on PBS or PpR24 fed aphids (Fisher's exact test p-value = >0.9999).

	Insects dead	**Insects alive**	**Marginal Row Totals**
Control	3	7	**10**
PpR24	3	7	**10**
Marginal Column Total	**6**	**14**	**20**

Table 6.3: Mortality results for *Orius laevigatus* ingestion of PpR24-fed *M. persicae*. Statistically higher mortality was observed in *O. laevigatus* predating on PpR24-fed aphids (Fisher's exact test p-value = 0.0325).

	Insects dead	**Insects alive**	**Marginal Row Totals**
Control	5	5	**10**
PpR24	10	0	**10**
Marginal Column Total	**15**	**5**	**20**

6.4 Discussion

It is important to consider the potential non-target effects when developing novel forms of crop protection. In an IPM system, biopesticides are often used alongside beneficial natural enemies and pollinators. In glasshouses in particular, natural enemies are commonly applied to control aphid infestations therefore it is prudent to assess the effects of PpR24 on common commercial aphid natural enemies. This Chapter has explored the direct effects of PpR24 on *Orius laevigatus*, *Aphidius colemani* and *Macrolophus pygmaeus* by simulating likely routes of exposure in a crop environment. The results found that the natural enemies varied in response to PpR24 depending on the method of exposure to the bacterium.

6.4.1 Evaluation of PpR24's non-target effects

Both *O. laevigatus* and *A. colemani* were negatively effected by PpR24 therefore it could be inferred that that PpR24 may have undesirable non-target effects if used in a crop system with certain beneficial species. However, *M. pygmaeus* insects showed no significant differences in survival over 72 hours in any of the topical, residual or oral exposure assays to PpR24, which may indicate that *M. pygmaeus* would be suitable for use alongside *P. poae* PpR24 in an IPM system. Although detrimental effects were observed in *O. laevigatus* and *A. colemani*, it does not definitively rule out the application of the bacteria when the insects are present in the system. *Orius* nymphs tend to be more concealed about a plant, such as in flowers, and so may avoid contact with the bacterial spray. Furthermore, PpR24 had no significant effect on *A. colemani* mummy emergence. It may be possible to apply the bacteria as a spray treatment when the insects are in juvenile stages to accommodate both approaches to aphid control with minimal beneficial insect casualties.

Topical applications of PpR24 at aphid-lethal concentrations [9] simulated droplet contact if sprayed in a crop environment. Significantly higher natural-enemy mortality was observed in *O. laevigatus* and *A. colemani* than PBS or control treatments, but no significant effect was observed in *M. pygmaeus*. It is possible that this is an insect size-related effect. Adult *M. pygmaeus* are about 3-6mm in length whereas *O. laevigatus* and *A. colemani* are much smaller, at 1.4-2.4mm and 2-3mm respectively. As such, *O. laevigatus* and *A. colemani* may be more susceptible to lower doses of the bacteria.

A possible route of entry for the bacterium are the insect spiracles, holes in the cuticle used for respiration, and the toxins may perforate throughout the insect body from there. The Hamilton study [8] hypothesised that the wild-type PpR24's aphicidal ability is in part due to gut occlusion. It may be that PpR24 is also forming occlusions in the spiracles of the insects, preventing respiration. Spiracle-blocking is seen in several commercial insecticides and is also the mode of action for biopesticides *Beavaria bassiana* and *Lecanicillium muscarium* which proved harmful to *Orius* sp. after dipping in insecticide solutions [331]. However, in light of the evolutionary passages conducted in Chapter 3, where biofilm formation was associated with a reduction of virulence, biofilm-mediated aphid killing may not be as significant a factor in PpR24's mode of action as previously hypothesised.

Exposure to residues of PpR24 spray on excised pepper leaves resulted in a statistically significant difference in *A. colemani* mortality when comparing PBS and wild-type PpR24 treatments. No significant differences in mortality were seen for *O. laevigatus* and *M. pygmaeus*. Similar protocols assessing the residual activity of pesticides on natural enemies have also found *A. colemani* to be sensitive to residual contact [332, 306]. However, topically applying aphid mummies with PpR24 had no significant effect on wasp emergence. It is possible the hardened cuticle of the mummy prevents the bacteria affecting the developing wasp. However, whether there are any long-term effects on the emergent wasps remains to be seen.

Both *M. pygmaeus* and *O. laevigatus* are taxonomically related to aphids as Hemipterans [333], therefore it is possible that toxins produced by PpR24 may affect them in a similar way to aphids. However, oral ingestion of aphids fed on PpR24 showed no significant effect for *M. pygmaeus* but a statistically significant effect was observed in *O. laevigatus*. Again, this may be due to the smaller size of *O. laevigatus* as the larger *M. pygmaeus* may be able to withstand the ingested dose of the bacteria.

6.4.2 The insect immune system

It is possible that the differences in susceptibility to PpR24 between species is due to variation in the insect immune system. The immune system of the fruit fly, *Drosophila melanogaster*, has been widely studied and acted as a model species in understanding the immune responses and strategies in insects

[334] as immune pathways have been found to be highly conserved in flies, mosquitoes, bees, and beetles [335]. The insect immune system acts via a combination of humoral and cellular defence responses to combat invading pathogens and parasites. Humoral defence mechanisms include the production of antimicrobial peptides (AMPs), reactive oxygen species (ROS), cascades that regulate coagulation, and melanization of hemolymph. Cellular defences involve processes such as phagocytosis, nodulation and encapsulation [336, 121, 337, 338, 339]. Beneficial gut symbionts have also been shown to provide defence against pathogens and parasites [340].

Aphids rely heavily on bacterial symbionts in bacteriocytes and the haemocoel to provide protection from parasitoid and pathogen attack [335, 341]. The absence and reduction of several genes associated with the insect immune system, such as AMP genes, from the aphid genome may be indicative that aphids have a poorer immune system than other species. Alternatively, the loss of genes may be due to bacterial symbionts and host plant compounds performing protective functions, removing the need for the insect to produce costly immune functions [341, 342, 343].

It is interesting that the previous study [8] found no significant effects on non-target species investigated in contrast to the current study. The absence of a significant effect in *Apis mellifera* larvae was found to be due to the antimicrobial properties of the royal jelly, but another potential causal factor may be that the insects examined during the Hamilton study were holometabolous insects, insects that undergo complete metamorphosis with a pupal stage [339]. During metamorphosis, holometabolous insects experience drastic changes in their physiology and immunological profile [344]. Metamorphosing insects in the larval and pupal stages have been shown to have increased antibacterial immune protection in the mid-gut. This may be to provide extra protection from pathogens and parasites in their sessile, vulnerable state, but also may have evolved as an adaptive response to control microbiota during gut replacement [345, 346, 347]. For example, in *Galleria mellonella*, increased expression of immune effectors and antimicrobial peptides occurred during pupal development and was found to peak during the delamination of the larval gut [345].

Compared to holometabolous insects, the development of the immune system in hemimetabolous insects (insects that mature through moult stages) is far less studied [341, 337]. However, it has been speculated that microbial density and diversity inside the insect gut (which can confer beneficial services)

continually grows overtime [340], in contrast to the fluctuating density and diversity of holometabolous microflora, which have been observed to go extinct in some instances [345].

It is possible that the differing immune system expression in holometabolous and hemimetabolous insects during development is a causal factor in the discrepancy in insect susceptibility to PpR24 between the Hamilton and current study. Immune system factors peak in larval stages of insects, which may account for why the insects in the previous study were not significantly affected by PpR24 as only larvae were examined. However, after the pupal stage, immune system microbiota in the gut is reduced due to the physiologocal change. This may be why the adult *A. colemani* wasps in the current study were affected by PpR24. Even though, they are holometabolous, the wasps used in the study were freshly emerged from their pupal stage and therefore may have lower levels of beneficial gut microflora, making them more susceptible to PpR24.

6.4.3 Limitations of evaluating non-target effects

The experiments in this Chapter were performed using commercially reared natural enemies. Therefore the age of the study insects were unknown. This may be responsible for the deaths of the insects in the control treatments, thereby reducing the potential differences in mortality due to the treatments applied. In future, the use of laboratory cultured insects would enable the collection of natural enemies of a known age and prevent these difficulties from occurring.

Furthermore, mortality observed in these lab-based experiments may not accurately reflect the mortality in field environments. For instance, in lab bioassays investigating the effects of spinosad on *Orius insidiosus*, a significant mortality effect was seen suggesting it would be unsuitable for use in an IPM system, whereas in glasshouse and field trials, no significant effects were observed [320]. Lab-based experiments are unable to take into account field conditions that may affect biopesticide action, such as changes to humidity, light and temperature, but also the movement of the insects in such large areas.

6.4.4 Suggestions for further work

Going forward, it would be interesting to assess any sub-lethal, indirect effects of the bacterium on natural enemy efficacy, such as fecundity and prey location. As mentioned in Chapter 5, herbivore induced plant volatiles can play an important role in natural enemy prey location and pest repellency. For instance, *Orius* bugs have been seen to induce heightened emission of plant defence volatiles in sweet pepper which repelled the whitefly *Bemisia tabaci* and thrip *Frankliniella occidentalis*, as well as proving attractive to the whitefly parasitoid *Encarsia formosa* [348]. Bacterial induced plant volatiles have also been seen to act as kairomones and it would be interesting to explore whether the volatiles induced by PpR24 on the plant are synergistic with natural enemy prey location or antagonistic.

As only adults were tested in this study, it may be pertinent to examine the effects of PpR24 on juvenile beneficials, as well as examine potential sub-lethal effects PpR24 may induce. For instance, many chemical treatments have been known to affect insect fecundity and predator egg development, which may prove problematic for growers if they wish to establish the natural enemies in the system. Timing of natural enemy and biopesticide application would be key to an effective combination of aphid control methods [296].

Another potential avenue to peruse would be to see where the predators and parasitoids can act as vectors for PpR24, directly transferring the bacterium from aphid to aphid, as well as from plant to plant. *O. laevigatus* has previously been seen to effectively disseminate the entomopathogenic fungus *Lecanicillium longisporum* or *L. muscarium* when doused with the fungal conidia [349]. 98% of *M. persicae* that came into contact with leaf discs exposed to treated *O. laevigatus* became infected [349].

6.5 Conclusion

The non-target effects of PpR24 on *Orius laevigatus*, *Macrolophus pygmaeus* and *Aphidius colemani* varied with species and the route of exposure. In all instances, *M. pygmaeus* was unaffected by the bacterium. When topically applied, a statistically higher mortality in *O. laevigatus* and *A. colemani* was observed in insects treated with PpR24. Significantly higher mortality was also observed for *O. laevigatus* when allowed to feed on aphids fed on PpR24

as well as in *A. colemani* when left to inhabit leaves sprayed with PpR24.

Although a detrimental effect was seen in some instances, the potential for PpR24 to be used as a biocontrol agent alongside these natural enemies in an IPM system as should not be ruled out. No significant effect was observed on *A. colemani* mummy emergence when topically applied with PpR24. Juvenile *Orius* development stages spend most time concealed about the plant and therefore may not come into contact with the bacteria if applied as a foliar spray.

A fundamental issue with this study is that the mode of action for PpR24's aphicidal properties still remains unclear. Although PpR24 seems to have a significant effect on the mortality of *O. laevigatus* and *A. colemani*, the mode of killing remains unknown. As such, elucidating PpR24's mode of killing should be a priority in further research. As well as this, field trials are also necessary to fully understand how this bacterium affects these natural enemies.

Chapter 7

Method development - Imaging *P. poae in situ*

7.1 Introduction

The question of where the bacterium acts inside the aphids still remains to be answered to elucidate PpR24's mode of action. At the present time, there are two foremost hypothesis of PpR24's mode of action, firstly that it acts by occlusion in the aphid gut, and secondly that the bacterium invades the body and kills via toxin production. In the Paliwal study, attempts were made to visualise *P. poae* inside the aphid by tagging the bacterium with a fluorescent protein. Using GFP, CFP, and RFP *E. coli* construct strains, Paliwal was able to successfully move the genes into *P. poae* but unfortunately fluorescent expression was not observed. In the current study, two alternative methodologies were explored as pilot experiments in attempts to observe where *P. poae* acts in, or on, the aphid host.

7.2 Internal bacterial location

Haematoxylin and eosin (H & E) staining and cryosectioning were conducted following the protocol in Mitchel *et al.* [350], in an attempt to view the bacteria *in situ* (Figure 7.1). Aphids were sectioned into 10μm slices, exposing whole body cavity, and comparisons between treated and control aphids could be observed. However, although in part successful, significant disruption was caused to the aphid cuticle during the sectioning process therefore accurate

comparisons could not be made. That being said, there is definite potential to image the bacterium's location in the aphids in this manner. Simonet *et al.* [351] have successfully applied (H & E) sectioning to image bacteriocyte tissue in the pea aphid *Acyrthosiphon pisum*, where the bacteriocyte tissue appeared darker to the light pink aphid epithelial tissue.

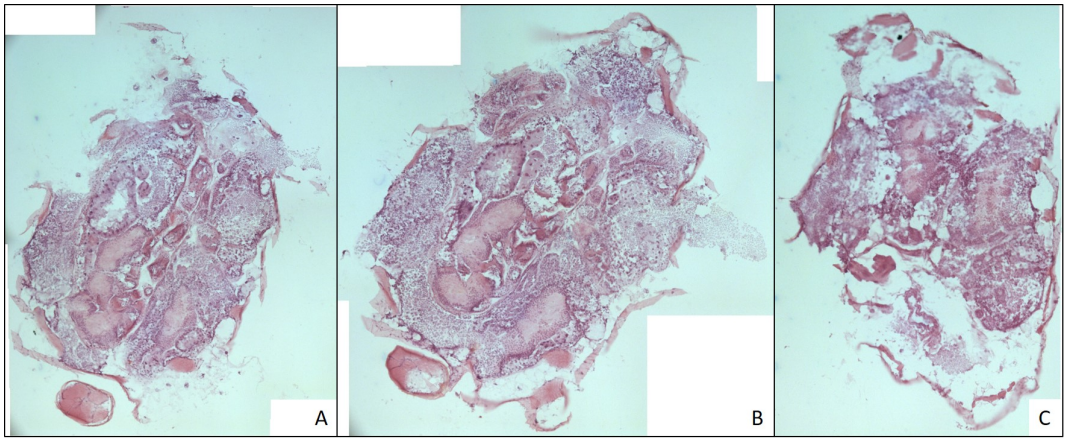


Figure 7.1: H & E-stained whole-aphid sections. Aphids were left to feed for 48 hours on artificial diet inoculated with PpR24 at 10^{-7} CFU mL^{-1} (Images A and B) Control sectioned aphids were fed on artificial diet sachets un-treated with PpR24 (Image C). At 48 hours, dead PpR24 fed aphids and control samples were collected and placed in liquid OCT and frozen in dry ice cooled ethanol. 10μm sections were collect using a cryostat and sections stained with Haematoxylin and Eosin Images were taken on an AxioSkop microscope.

7.3 External bacterial location

Scanning electron microscopy was also used in an attempt to locate bacteria on the external surfaces of deceased aphids (Figure 7.2). It is possible that the aphids encounter the bacteria from excreted honeydew deposits and transport it from host plant to host plant. Deceased aphids that were left to feed on treated sachets, as described above, were imaged using a FEI Quanta FEG 600 Scanning Electron Microscope equipped with a Quorum PP2000T Cryo Stage. Whole aphids were prepared by rapidly freezing the insects and maintaining them in a vacuum at approximately -130°C, coated in a conductive metal and water removed by sublimation before imaging at approximately -175°C [352]. Unfortunately this process did not result in a uniform coating on the aphids and this affected the clarity of the images taken. Another issue that arose

was the occurrence of ice crystals on the aphids, which were initially mistaken for bacteria. These crystals likely formed as the freezing process was not fast enough. No bacteria were observed on the external surfaces of the imaged aphids.

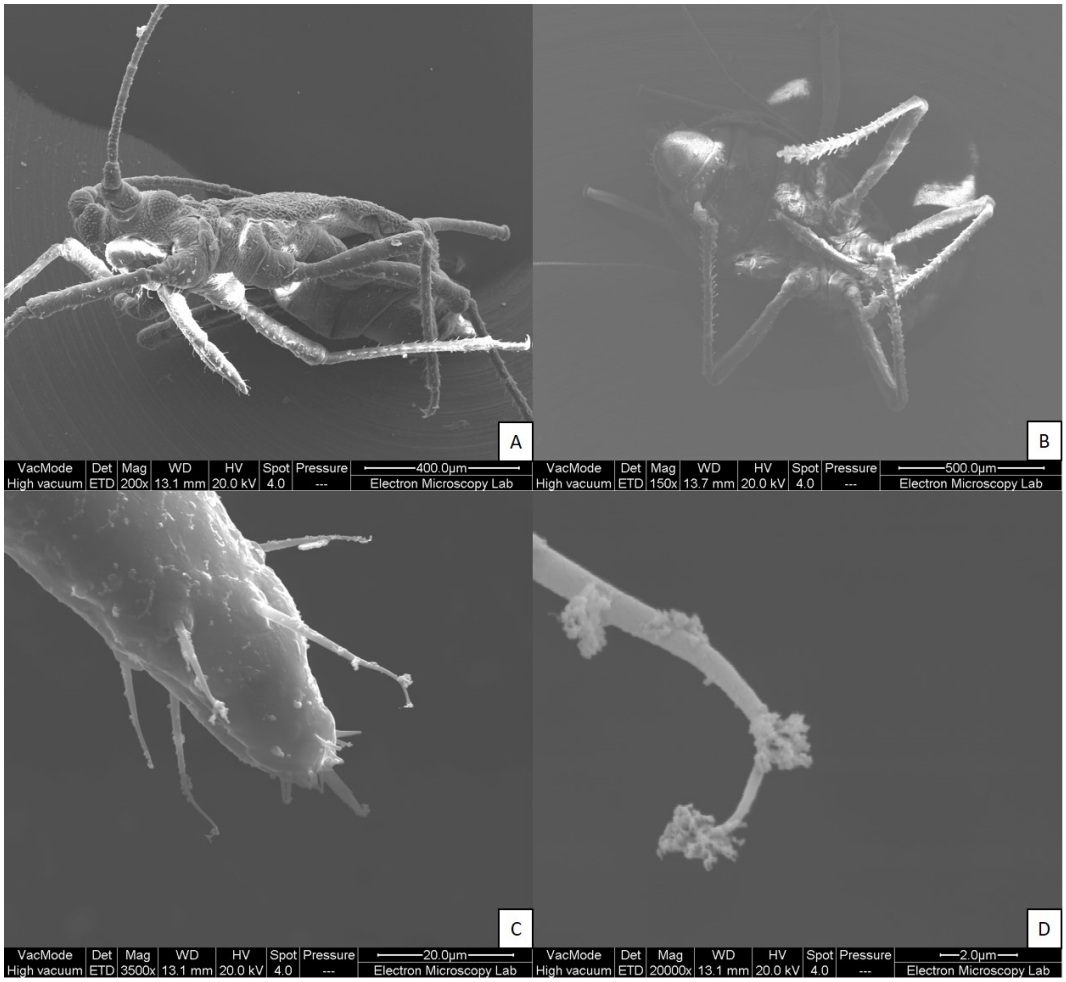


Figure 7.2: Green peach-potato aphids, *Myzus persicae*. Images taken using a FEI Quanta FEG 600 Scanning Electron Microscope equipped with a Quorum PP2000T Cryo Stage.

7.4 Future approaches to visualisation

In future investigations, immuno-gold tagging PpR24 cells combined with imaging sectioned aphids with an electron microscope may prove an effective means of localising the bacteria inside the aphid [353, 354].

Flourescent *in situ* hybridization (FISH) may be another suitable method for imaging *P. poae in situ*. FISH utilises specific rRNA-targeted gene probes

with fluorescent dyes to detect and localise bacteria [355]. FISH has been applied to detect bacteria and endosymbionts in arthropods, for example, visualising endosymbionts in lice to better understand the host-endosymbiont relationship [356]. FISH also offers the advantage of allowing the whole organism view of where the bacteria is, making it a useful tool in characterising internal habitats of bacteria. With this aim, a combination of cryosectioning and FISH was successfully applied to investigate the bacteria inhabiting the gut of Collembola arthropods (Springtails) [355]. With the addition to visualising the action of *P. poae* inside the aphid, combined cryosectioning and FISH imaging [351] may also elucidate whether the presence of aphid endosymbionts affect PpR24.

Chapter 8

General Discussion and further work

8.1 General Discussion

The overall aim of this research was to investigate whether experimental evolution could be applied to improve the efficacy of *Pseudomonas poae* PpR24 as an aphid biocontrol agent and to better understand how PpR24 affects aphids and non-target insects. The findings presented in this Thesis may prove useful in designing IPM systems in glasshouses with PpR24.

Wild-type isolates of PpR24 were serially passaged in environments intended to select for either aphid-virulence or biofilm formation, after which final passaged isolates were observed for different phenotypes and trade-offs between traits (Chapter 3). Aphid virulence was not improved but a significantly strong biofilm-forming isolate evolved at a cost to aphid virulence. Whole genome sequencing and variant calling analysis identified two point mutations plausibly responsible for the biofilm phenotype and reduction in aphid virulence (Chapter 4). Identification of volatile organic compounds emitted by the wild-type *P. poae* PpR24 was carried out to elucidate the deterrent effect seen in the Paliwal study, as well as further examine phenotypic differences between the wild-type and biofilm-forming isolate (Chapter 5). Finally, further assessment of PpR24's interactions with non-target insects was carried out, investigating the lethal effects of PpR24 on commercially used aphid natural enemies (Chapter 6).

8.1.1 Experimental evolution of *Pseudomonas poae* PpR24

The first Chapter of this study attempted to improve the efficacy of PpR24 as a biocontrol agent without directly modifying PpR24's genome. By employing an experimental evolution approach, an investigation into whether an increase in the virulence of PpR24 from 70% aphid mortality in 48 hours could be achieved, either by higher total number of dead aphids or faster killing. Whether PpR24 could evolve biofilm-formation was also investigated on the hypothesis that biofilm aggregations may improve PpR24's foliar colonisation and enhance aphid mortality by gut occlusion.

No statistically significant changes to aphid mortality were observed between the wild-type and final passage evolved isolates from which we can infer that no improvements to the rate of aphid killing were made. However, one final-passage isolate, PpR24b4, showed statistically stronger biofilm formation than the other biofilm-passaged isolates. An examination into the trade-offs between passaged isolates and the wild-type PpR24 revealed that biofilm-passaged isolates passaged through broth microcosms had altered phenotypic properties to the wild-type and aphid passaged isolates. Biofilm-passaged isolates exhibited reduced growth, were less motile in agar assays and, of particular poignance, were less effective at killing aphids. In addition, no improvement to plant colonisation was observed in biofilm or aphid-passaged isolates.

It is possible that no phenotypic differences between the aphid-passaged and wild-type PpR24 isolates were observed due to the process of growing the recovered isolates on agar plates between passages, which may have lessened the selective force for survival inside the aphid. As a consequence, only ten passages may have been insufficient for improved virulence to evolve. Alternatively, the aphid functions as a viable host for the bacterium as it is able to successfully replicate inside the aphid. The aphid may also function as an effective vector for PpR24. Dr Paliwal's study hypothesised that ingested PpR24 move through the digestive tract and replicate in the insect gut, resulting in the build up of aphicidal toxins and consequent aphid demise. Stavrinides *et al.* [122] made a similar hypothesis addressing the virulent properties of the plant pathogen *P. syringae* PsyB728a to the aphid *Acyrthosiphon pisum*. PsyB728a is capable of replication in the aphid gut and once large enough populations are reached, the bacterium is expelled and dispersed onto a plant

via aphid honey dew. To the bacterium, the aphid functions as a viable host and vector, alongside its usual plant environment, where it can reproduce and disperse into a new environment efficiently. It may be a similar scenario in the case of PpR24, where the bacterium is already at equilibrium between replication (with the build up of aphicidal toxins) and transmission. Any increase in virulence may come at the detriment to the bacterium as the aphid host may be prematurely killed before the bacterium can be expelled and transmitted to a new host.

With regards to the biofilm-forming phenotype, it is possible that the reduced virulence may be due the isolate's poorer growth. Replication and occlusion in the aphid gut was speculated to be a potential mode of action in PpR24 virulence. The reduced growth of the strong biofilm forming isolates may impair the isolate's ability to replicate in the aphid gut and consequently not reach high enough populations to have a virulent effect. Furthermore, the aphid gut is home to a variety of endosymbionts, which in some instances have anti-microbial properties (which will be discussed in Section 8.2.2). It is possible that the biofilm-passaged isolates are out competed by the endosymbionts due to their reduced motility and poorer growth. The reduced motility associated with cell aggregation may also be a causal factor in the reduction aphid virulence as it may impair the bacterium's ability to move about the aphid, whether to reach suitable areas in the aphid gut for occlusion, or actively invade the insect with aphicidal toxins. In light of the biofilm-passaged isolates and strong biofilm former PpR24b4's poor performance in aphid virulence, it may be inferred that the production of virulent toxins by the wild-type PpR24 is the more likely mode of action than aggregation and occlusion in the aphid gut.

Although academically a success in evolving a phenotypically different isolate of PpR24, in terms of practical applications as a biocontrol agent reducing growth and isolate virulence was less than ideal. However, it is possible that the derived isolates still have value as plant growth promoters. Hamilton's study revealed PpR24 promotes host plant growth and the biofilm form of lifestyle is common among plant growth promoting rhizobacteria, including several Pseudomonads. As *P. poae* is effectively disseminated as a soil drench, there is potential for it to be applied as a PGPR to boost crop yields which could be investigated further in future work.

8.1.2 Genome sequencing of wild-type and passaged isolates

To further understand the phenotypic changes in the passaged isolates from Chapter 3, hybrid assembly and variant calling analysis was conducted to identify mutations differing from the wild-type PpR24 in the strong biofilm forming isolate, PpR24b4, and an aphid-passaged isolate, PpR24a1. No discrepancies were found between isolate PpR24a1 and the wild-type, corroborating with the results of the phenotypic analysis in Chapter 3 that no significant phenotypic or genomic changes occurred as a result of the aphid-passages. On the other hand, two missense point mutations were found in isolate PpR24b4. One in the gene *cheB*, homologous to *wspF* in Pseudomonads, which encodes for a methylesterase in the Wsp chemosensory pathway. The other mutation occurred in *barA*, the homologue of which in Pseudomonads is *gacS*, which encodes the sensor kinase in the two-component GacS/GacA regulatory system.

Both the the Gac and Wsp pathways have been seen to be instrumental in phenotype switching between a planktonic and biofilm lifestyle and therefore are likely to contribute to biofilm formation in PpR24b4. The two-component GacS/GacA regulatory system is responsible for secondary metabolite and extra-cellular protein production and has been well studied in Pseudomonads [222]. The activation of GacA by GacS, leads to the expression of small RNAs, such as RsmY and RsmZ, which regulate the repressor RsmA. When high levels of RsmA are present in a cell, traits associated with motility and virulence are promoted. At low RsmA levels, biofilm associated genes and EPS production are expressed. In fluorescent Pseudomonads, mutations in *gacS* have been seen to reduce bacterial virulent properties, as well as motility.

Two-component systems, such as the Gac system, act in parallel to c-di-GMP regulatory pathways that contain diguanylate cyclases (DGCs), enzymes that synthesise (c-di-GMP). The Wsp pathway, is one such pathway, containing the response regulator WspR (a DGC). WspF regulates the activity of WspR, preventing the production of c-di-GMP and enabling the expression flagellar genes and traits associated with motility, such as in the wild-type SBW25. However, mutations in a loss-of-function in the WspF methylesterase allow for the continuous activation of WspR and the production of c-di-GMP. High levels of c-di-GMP result in the repression of motile traits, such as flagellar and virulence genes, and the expression of extracellular polysaccharides and

adhesive substances, typical of the biofilm lifestyle. For instance in the case of *Pseudomonas fluorescens* SBW25, mutations in WspF resulted in the repression of FleQ, the activation of the cellulose-producing Wss operon and the over-expression of adhesive substances which resulted in a Wrinkly Spreader, biofilm-forming phenotype.

In the context of the extensive literature in biofilm formation, it appears likely that both the mutations observed in PpR24b4 are important factors in biofilm formation and may explain the phenotypic differences in motility and virulence observed between PpR24b4 and the wild-type in Chapter 3. Aphicidal toxins identified in the Paliwal study may also be regulated by the two-component GacS/GacA system and as a consequence may no-longer be produced in favour of biofilm traits, which may account for the reduction in aphid virulence in biofilm passaged isolates. Similarly, the GacS/GacA system has been implicated in the production of anti-microbial production and volatile organic compounds (VOCs) in bacteria. Therefore the differences in VOC emissions between PpR24 and PpR24b4 in Chapter 5 may also be due to transition to a sessile phenotype, where the production of such secondary metabolites are no-longer necessary, or ceased to cover the fitness cost of the biofilm lifestyle (Figure 8.1).

It appeared biofilm formation was at least a two stage process as a loss of pigmentation preceded pellicle formation at the air-liquid interface. This theory was confirmed by the subsequent Capano study, which detected the mutation in *gacS* was present from the first evolutionary passage, whereas the mutation in *wspF* only evolved in passage seven, coinciding with the emergence of strong biofilm production.

Phenotype switching between a motile and biofilm lifestyle is a well-coordinated process involving several complex regulatory pathways and different pathways containing DGCs that activate at specific stages during the transition to a biofilm state [222]. It is thought that TCSs control the pace of biofilm development by regulating DGC activation [357]. WspR has been seen to be active during the microcolony formation stage of biofilm development, which corroborates with the thick biofilm produced from passage 7 by PpR24b4 [222]. However, the GacS/GacA TCS is thought to be active in earlier stages of initial bacterial surface attachment and regulate c-di-GMP production by the DGC SadC via RsmA [222]. Therefore the mutation in *gacS* may affect the levels c-di-GMP by the activation of SadC, initiating bacterial attachment for sub-

sequent colony formation mediated by WspR. However, it is unclear whether the mutation in *wspF* is dependent on the presence of the *gacS* mutation, or indeed whether both are necessary for biofilm formation. Gene knock-out mutagenesis or allele replacement approaches introducing the mutations into the wild-type PpR24 may provide confirmation that these genes are responsible for the biofilm phenotype.

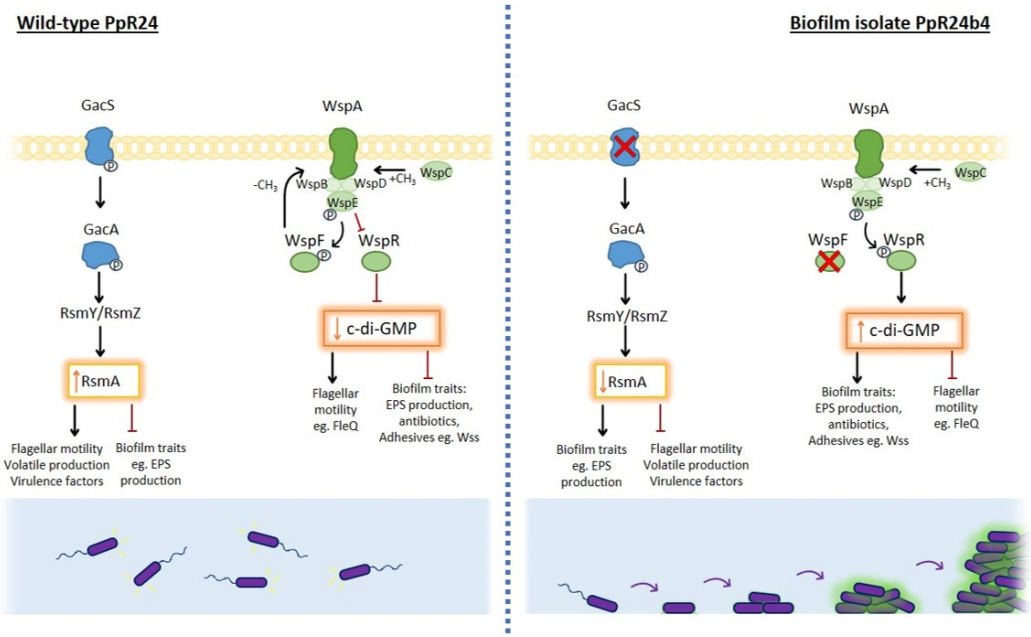


Figure 8.1: The hypothesised consequences of the PpR24b4 mutations in the Gac and Wsp for the transition between a planktonic and biofilm lifestyle, based on the *P. aeruginosa* and SBW25 model systems. In the planktonic wild-type PpR24, the GacS/GacS TCS, phosphorylation of GacA by GacS promotes the transcription of small RNAs, RsmY and RsmZ. High levels of RsmA regulates the repression of biofilm traits, such as EPS production and regulation of c-di-GMP synthesis by the diguanylate cyclase (DGC) SadC, promoting flagellar expression and the production of virulence and volatile compounds [225, 357, 358, 222]. In the Wsp chemosensory pathway, a functioning WspF methylesterase removes the methyl group from the methyl-accepting chemotaxis protein, WspA, preventing the activation of the DGC, WspR, and the synthesis of c-di-GMP in the cell. Consequently, flagellar genes are expressed and biofilm associated traits are not [206].
In the biofilm-forming phenotype (PpR24b4), RsmA is sequestered by RsmY and RsmZ, reducing RsmA levels in the cell thus allowing the expression of biofilm determinants, such as EPS and c-di-GMP production, and the repression of genes associated with motility and virulence. A loss-of-function in WspF results in the constitutive activation of WspR and the production of c-di-GMP. As a result, flagellar genes are repressed and the production of biofilm traits, such EPS and adhesive substances, promoted.
(This is a simplified version of the complex, coordinated web of TCSs and DGSs involved in the transition from a motile to sessile lifestyle, focussed on the pathways directly associated with the mutations detected in the variant calling analysis, indicated by red crosses.)

8.1.3 Examination of PpR24's volatile compounds and their effects on *M. persicae* settling behaviour

This study has furthered our understanding of *P. poae* PpR24's aphid deterrent properties by identifying volatile organic compounds emitted by the bacterium. An examination of headspace volatiles emitted by PpR24 in a broth environment identified several compounds associated with plant growth promotion and anti-microbial properties. No changes from the wild-type volatile emissions were detected in isolate PpR24a1, which further corroborated the findings in Chapters 3 and 4 that no significant phenotypic or genotypic changes occurred during the aphid-passages. However, biofilm-forming isolate PpR24b4 had reduced levels of 1-decene, 1-nonene, 1-undecene and dimethyl sulphide, and elevated levels of methanethiol and 2-methylpentane when compared to the wild-type PpR24.

Speculatively, it is possible the changes in VOC emissions are related to regulatory changes induced by TCS, such as GacS/GacA, altering enzyme activity during the phentotype switching from motilty to sessility. One possible pathway involved may be the membrane MeSH-dependent dimethyl sulphide (DMS) production pathway (Mdd), where DMS is produce by the methylation of methanethiol (MeSH) via the membrane methyltransferase, MddA. MddA is abundant in soil bacteria and is well conserved across *Pseudomonas* species [359, 360, 361]. In *Pseudomonas deceptionensis* $M1^T$, methionine is converted to MeSH by the Met gamma lyase enzyme (MegL), which is subsequently methylated to DMS [359]. DMS can act as a signalling molecule and carbon and energy source for bacteria [362], therefore it is possible that higher emissions are detected in the active, motile state of the wild-type due to the need for more readily available energy reserves. On the other hand, in a sessile state, the production of DMS may be reduced in favour of biofilm factors, such as EPS production, which may explain the increase of methanethiol emissions as less DMS is being methylated.

Previous research found a deterrent effect to be present when aphids were exposed to volatiles gathered from plants sprayed with the PpR24. The majority of volatiles detected from PpR24-sprayed plant headspaces in the current study were identified as green leaf volatiles (GLVs) and herbivore induced plant volatiles (HIPVs) that act as kairomones for insect predators and parasitoids. Foliar application of plants with different bacterial isolates (PpR24, PpR24a1

or PpR24b4) or control sprays resulted in no significant differences in volatile compounds detected in the plant headspace. It is possible that the GLVs and HIPVs are deterring the aphids. However, an examination into the whether the evolved isolates, PpR24a1 and PpR24b4, retained their aphid-deterrent properties found the derived isolates no longer repelled aphids from a sprayed plant. Alterations in the bacterium's volatile emissions may be causal in the loss of repellency, however visual cues may also play a vital part in aphid host plant choice.

8.1.4 Mortality of natural enemies

Macrolophus pygmaeus insects were not statistically affected by any of the PpR24 treatments. However, results suggested that *O. laevigatus* insects were affected by oral ingestion of bacteria-treated aphids and topical contact with *P. poae*. Adult *A. colemani* were also statistically affected by topical applications of PpR24 and exposure to PpR24 residues on leaf surfaces. These findings suggest there are undesired lethal effects of *P. poae* PpR24 on non-target beneficials in some exposure scenarios. However, topical application of PpR24 to mummified aphids was not statistically detrimental to wasp emergence.

The robustness of the results must be called into question. As insects were ordered in, it cannot be guaranteed that all mortality observed in sample insects was due to the action of the bacteria and therefore the age of the insects may not have been completely the same, thus some may have simply died of old age. Repeating the experiment with insects reared on site may solve such limitations.

8.2 Suggestions for further work

Several concepts for further research into understanding *Pseudomonas poae* PpR24 directly relevant to the experiments conducted in this Thesis have been discussed in the previous Chapters. The need to ascertain where the bacterium acts in, or on, the aphid is of particular importance to elucidate whether the PpR24's virulent properties are due to occlusion in the aphid gut or invasion of the body with the release of aphicidal toxins, as discussed in Chapter 7. Sections 8.2.1 to 8.2.4 suggest potential directions for future research in understanding PpR24 and it's potential use as a crop protection

agent.

8.2.1 *P. poae's* sub-lethal effects on beneficial insects

This study focused on the short-term, lethal effects of *Pseudomonas poae* PpR24 on aphid natural enemies. It may be valuable in future research to investigate longer-term and sub-lethal effects of PpR24 on beneficial insects, such as effects on fecundity [249].

As the volatiles produced by *Pseudomans poae* have been seen to affect aphid behaviour when applied to a plant, it is possible that natural enemy behaviour may also be affected. Several of the volatile compounds identified from bacteria-plant assays in Chapter 5 have been seen to act as kairomones in insect host/prey location. Olfactometer choice-apparatus could be used to examine the effects of *P. poae* on natural enemy prey detection in a range of scenarios [249, 363, 364]. For example, olfactometers were used to assess how the pesticide deltamethrin affects *Aphidius ervi* orientation to host-infested plants [330]. Parasitic wasps in particular are reliant on kairomones to locate viable hosts. It is possible that the presence of *P. poae* on the plant may affect parasitoid host searching behaviour [259].

The effects of PpR24 on adult bees may also be of interest. Bumble bees, *Bombus terrestris*, are commercially used as crop pollinators in glasshouse systems [365] and therefore may be exposed to PpR24 directly by spray application, or indirectly via plant nectar or pollen, as PpR24 can enter the plant internal system and has been shown to be present on internal leaf surfaces [9](although the extent that it can spread through the plant has not been studied). As discussed in Chapter 6, previous research [8] found PpR24 had no statistically significant effects on the survival of *Apis mellifera* larvae, thought to be due to the antimicrobial properties of the royal jelly used in the nutrition of the larvae. However, whether PpR24 has lethal or sub-lethal effects on adult bees remains to be seen. As well as observing bee mortality, measurements in bee activity, distance travelled by bees and colony size may prove insightful in assessing whether PpR24 is suitable for use alongside bees pollinators. For example, pressure sensors and camera set-ups can record the departure and return of bees at the hive [366] and methods such as passive radio frequency identification (RFID) tags [367] or applying fluorescent powder to bees as they leave the hive and observing where the powder is present in the crop, can be

used to observe monitor effects on bee activity as indicators of bee health. Furthermore, it could be investigated whether bees are capable of vectoring the bacterium as it pollinates flowers in the crop.

8.2.2 The effects of endosymbionts

Endosymbionts are micro-organism inhabitants of the aphid gut that have been co-evolving a mutualistic relationship with their hosts for about 160-280 million years. Endosymbionts can be obligate or facultative and many have been seen to play important roles in aphid ecology [368]. For instance, aphids are unable to acquire key nutrients and essential amino acids from their phloem diet and instead rely on obligate microbes, like *Buchnera aphidicola*, to produce the otherwise unobtainable nutrients. In the case of pea aphids, *A. pisum*, reared on broad beans, 90% of essential amino acids were produced by bacterial symbionts [369].

Facultative symbionts are not essential for aphid survival but can affect their aphid hosts in a variety of ways, such as providing tolerance to heat, protection from predators and pathogen resistance. *Hamiltonella defensa* and *Serratia symbiota* are two such symbionts well studied for their ability protect aphids from parasitoid attack. Isolates of *H. defensa* have been seen to disrupt the development of parasitoids inside the aphids and alter ovipositing wasp behaviour, such as causing the the wasp to oviposit more than once in a host and in some case avoid the aphid host where the endosymbiont is present all together. Endosymbionts have also been seen to benefit aphids by providing pathogen resistance and so may reduce *P. poae*'s aphicidal efficacy. *A. pisum* infected with the symbiont *Regiella insecticola* showed increased resistance to the fungal entomopathogen *Pandora neoaphidis* [197, 370]. The facultative endosymbionts *Ricettsia* sp. and *Ricketsiella* sp. have also been seen to convey fungal pathogen resistance [371]. However, symbionts do not provide uniform protection from all pathogens, for instance *Regiella insecticola* did not reduce aphid mortality when exposed to *Beauveria bassiana*, and therefore may not provide protection from PpR24. Furthermore, symbiont-conferred protection has been seen to impair aphid immune system function in the presence of fungal and Gram-negative pathogens [197] and in the case of *A. pisum* genes common in insect immune function were missing [335]. Therefore investigating the effects of endosymbionts on PpR24 merits further research to fully understand

the ecological efficacy of PpR24 as a biological control agent.

8.2.3 PpR24 toxins in transgenic crops

As stated in Chapter 1, Section 1.4.1, several toxin genes were identified in PpR24 encoding for proteins belonging to the Tc insecticidal toxin complex: two TcA-like (TcaA1, TcaB1), one TcB-like (TcaC1) and one TcC-like (TccC2) [9]. It is possible that these toxins may be applicable for use in transgenic crops. The majority of transgenic crops that confer insect resistance express Cry toxins from *Bacillus thuringiensis* (Bt) [83], therefore the Tc genes of PpR24 may provide a useful alternative in case resistance to Cry toxin expression evolves in pest species [102].

8.2.4 PpR24 effects on sweet pepper flavour

Finally, in Chapter 5 foliar application of PpR24 was shown to alter the levels of volatile emissions detected in the sweet pepper plant headspace, possibly mediating an aphid deterrent effect. Changes in volatiles have been associated with changes in flavour [372, 373], for instance during fruit ripening [374]. It would be prudent to assess whether the application of PpR24 on sweet pepper plants affects fruit flavour as well as the plant volatile emissions. Even if PpR24 is effective as a biocontrol agent, it would ultimately be worthless to growers if the flavour quality is impaired.

8.3 Conclusions and applications in an integrated system

The work presented in this study further supports evidence that PpR24 has potential for commercialisation as an aphid biocontrol agent in an IPM system. As attempts to evolve improved aphid virulence were unsuccessful, the wild-type *Pseudomonas poae* PpR24 still remains the best candidate for an aphid biocontrol agent. However, derived biofilm forming isolates may still have value as plant growth promoters [169]. As no improvement to aphid-killing was made, future work in sweet pepper systems may focus on the efficacy of systemic aphid control when *P. poae* is applied as a soil drench. It is unclear whether such an application technique would still deter aphids from a

plant or whether it would affect beneficial insects. However, although a foliar spray application may not be ideal in sweet pepper cropping systems, foliar application of *P. poae* may be applicable to other protected edibles, such as strawberries.

PpR24 could be used effectively as a foliar spray in both curative and preventative strategies, directly reducing aphid populations, but also deterring target pests from crop plants. However, to minimise the likelihood of aphids developing resistance to PpR24 treatment, it may be best if used in rotation with other pest control methods. This study found the presence of PpR24 on a sweet pepper plant was able to deter aphids to a control plant where the bacteria was not present. Furthermore, sprayed plants emitted green leave volatiles used in plant defence that may act to prime nearby plants to potential aphid threats. If used in a system with banker-plant buffer zones that remain un-sprayed by the bacteria, it may be that any pests are deterred from the crop to the banker plants. Pre-emptive spraying of crops before serious infestations occur may push pests onto banker plants acting as sink-zones, minimising crop losses. Such a method may be combined with natural enemies established in the banker plant to feed on the displaced aphids.

P. poae may be suitable for use alongside aphid natural enemies to ensure maximum aphid control. Of the insects used in this study, *Macrolophus pygmaeus* may be the most applicable aphid predator for use in conjunction with *P. poae*. Carefully timed spray applications may also mean *P. poae* is suitable for use with other parasitoids and predators, although more research must be conducted into this. As developing wasp emergence was not affected by topical applications of the bacteria, it may possible to spray crops when parasitoids are developing as mummies. Furthermore, juvenile *O. laevigatus* may avoid direct contact with *P. poae* spray as early life-cycle stages are spent in more concealed areas of the plant and thus are less likely to directly encounter the bacteria. Timing bacterial application with the life-cycle of introduced natural enemies may reduce losses of other, non-target beneficials present in the crop as pollinators and to control other pest species.

Up to this point, all investigations involving PpR24 have taken place in lab-based environments. Rigorous field trials would need to be conducted to fully understand the potential of *P. poae* PpR24 as a biocontrol agent and to ensure it is in line with biopesticide regulations, such as maximum residue levels left on a crop [375]. In addition, further steps need to be taken to improve *P.*

poae's shelf-life as a product.

In conclusion, *Pseudomonas poae* PpR24 still shows a great deal of promise as a biocontrol agent and, as an anecdotal note, the overall impression from individuals and organisations encountered over the course of this PhD suggests that the development of a safe and effective novel biopesticide agent, such as *Pseudomonas poae* PpR24, would be well-received by growers and the crop protection industry.

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Appendices

Appendix A

Chapter 2 Additional Data

Table A.1: Recipe for aphid Mittler diet. Compounds are added to 100ml of water with 15g of dissolved sucrose in the order and quantities presented.

No.	Compound	mg
1	Di-Potassium hydrogen orthophosphate	750
2	Magnesium sulphate	123
3	Tyrosine	40
4	L-Asparagine hydrate	550
5	L-Aspartic acid	140
6	L-Tryptophan	80
7	L-Alanine dextro-rotary	100
8	L-Arginine monohydrochloride	270
9	L-Cysteine hydrochloride, hydrate	40
10	L-Glutamic acid	140
11	L-Glutamine	150
12	Glycine	80
13	L-Histidine, free base	80
14	L-Isoleucine (allo free)	80
15	L-Leucine	80
16	L-Lysine-monohydrochloride	120
17	L-Methionine	40
18	L-Phenylaline	40
19	L-Proline	80
20	L-Serine	80
21	L-Threonine	140
22	L-Valine	80
23	L-Ascorbic acid (Vitamin C)	100
24	Aneurine hydrochloride (Vitamin B)	2.5
25	Riboflavin	0.5
26	Nicotinic acid	10
27	Folic acid	0.5
28	(+)-Pantothenic acid (calcium salt)	5
29	Inositol (meso) inactive	50
30	Choline chloride	50
31	Ethylenediameinetetra acetic acid	1.5
32	Fe (III)-Na chelate pure	1.5
33	EDTA Zn-Na2 chelate pure	0.8
34	EDTA Mn-Na2 chelate pure	0.8
35	EDTA Cu-Na2 chelate pure	0.4
36	Pyridoxine hydrochloride (Vitamin B6)	2.5
37	D-Biotin-crystalline	0.1

Appendix B

Chapter 3 Additional Data

B.1 Analysis of trade-offs between isolates

B.1.1 Trade-off in aphid virulence

Table B.1: Complete pairwise differences for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment aphid virulence. Kruskal-Wallis test p-value = <0.0001.

Dunn's multiple comparisons test	**Mean rank diff.**	**Sign.?**	**Summary**	**Adjusted P-Value**
Control vs. WT	-119.6	Yes	**	0.0014
Control vs. PpR24a1	-132.4	Yes	***	0.0001
Control vs. PpR24a2	-133.2	Yes	***	0.0001
Control vs. PpR24a3	-111.5	Yes	**	0.0058
Control vs. PpR24a4	-116	Yes	**	0.0027
Control vs. PpR24a5	-124.6	Yes	***	0.0006
Control vs. PpR24a6	-116.3	Yes	**	0.0025
Control vs. PpR24a7	-110.6	Yes	**	0.0067
Control vs. PpR24a8	-91.5	No	ns	0.2802
Control vs. PpR24a9	-107.2	Yes	*	0.0118
Control vs. PpR24a10	-102.3	Yes	*	0.0404
Control vs. PpR24b1	-15.17	No	ns	>0.9999
Control vs. PpR24b2	-9	No	ns	>0.9999
Control vs. PpR24b3	-35.33	No	ns	>0.9999

Control vs. PpR24b4	-31.61	No	ns	>0.9999
Control vs. PpR24b5	-76.22	No	ns	0.9138
Control vs. PpR24b6	-26.11	No	ns	>0.9999
Control vs. PpR24b7	-25	No	ns	>0.9999
Control vs. PpR24b8	-26.39	No	ns	>0.9999
Control vs. PpR24b9	-107.1	Yes	*	0.012
Control vs. PpR24b10	-72.17	No	ns	>0.9999
WT vs. PpR24a1	-12.89	No	ns	>0.9999
WT vs. PpR24a2	-13.67	No	ns	>0.9999
WT vs. PpR24a3	8.056	No	ns	>0.9999
WT vs. PpR24a4	3.556	No	ns	>0.9999
WT vs. PpR24a5	-5.056	No	ns	>0.9999
WT vs. PpR24a6	3.278	No	ns	>0.9999
WT vs. PpR24a7	9	No	ns	>0.9999
WT vs. PpR24a8	28.06	No	ns	>0.9999
WT vs. PpR24a9	12.39	No	ns	>0.9999
WT vs. PpR24a10	17.24	No	ns	>0.9999
WT vs. PpR24b1	104.4	Yes	*	0.0183
WT vs. PpR24b2	110.6	Yes	**	0.0067
WT vs. PpR24b3	84.22	No	ns	0.3354
WT vs. PpR24b4	87.94	No	ns	0.2044
WT vs. PpR24b5	43.33	No	ns	>0.9999
WT vs. PpR24b6	93.44	No	ns	0.095
WT vs. PpR24b7	94.56	No	ns	0.081
WT vs. PpR24b8	93.17	No	ns	0.0988
WT vs. PpR24b9	12.5	No	ns	>0.9999
WT vs. PpR24b10	47.39	No	ns	>0.9999
PpR24a1 vs. PpR24a2	-0.7778	No	ns	>0.9999
PpR24a1 vs. PpR24a3	20.94	No	ns	>0.9999
PpR24a1 vs. PpR24a4	16.44	No	ns	>0.9999
PpR24a1 vs. PpR24a5	7.833	No	ns	>0.9999
PpR24a1 vs. PpR24a6	16.17	No	ns	>0.9999
PpR24a1 vs. PpR24a7	21.89	No	ns	>0.9999
PpR24a1 vs. PpR24a8	40.94	No	ns	>0.9999

PpR24a1 vs. PpR24a9	25.28	No	ns	>0.9999
PpR24a1 vs. PpR24a10	30.13	No	ns	>0.9999
PpR24a1 vs. PpR24b1	117.3	Yes	**	0.0021
PpR24a1 vs. PpR24b2	123.4	Yes	***	0.0007
PpR24a1 vs. PpR24b3	97.11	No	ns	0.0557
PpR24a1 vs. PpR24b4	100.8	Yes	*	0.0318
PpR24a1 vs. PpR24b5	56.22	No	ns	>0.9999
PpR24a1 vs. PpR24b6	106.3	Yes	*	0.0134
PpR24a1 vs. PpR24b7	107.4	Yes	*	0.0112
PpR24a1 vs. PpR24b8	106.1	Yes	*	0.0141
PpR24a1 vs. PpR24b9	25.39	No	ns	>0.9999
PpR24a1 vs. PpR24b10	60.28	No	ns	>0.9999
PpR24a2 vs. PpR24a3	21.72	No	ns	>0.9999
PpR24a2 vs. PpR24a4	17.22	No	ns	>0.9999
PpR24a2 vs. PpR24a5	8.611	No	ns	>0.9999
PpR24a2 vs. PpR24a6	16.94	No	ns	>0.9999
PpR24a2 vs. PpR24a7	22.67	No	ns	>0.9999
PpR24a2 vs. PpR24a8	41.72	No	ns	>0.9999
PpR24a2 vs. PpR24a9	26.06	No	ns	>0.9999
PpR24a2 vs. PpR24a10	30.91	No	ns	>0.9999
PpR24a2 vs. PpR24b1	118.1	Yes	**	0.0019
PpR24a2 vs. PpR24b2	124.2	Yes	***	0.0006
PpR24a2 vs. PpR24b3	97.89	Yes	*	0.0496
PpR24a2 vs. PpR24b4	101.6	Yes	*	0.0282
PpR24a2 vs. PpR24b5	57	No	ns	>0.9999
PpR24a2 vs. PpR24b6	107.1	Yes	*	0.0119
PpR24a2 vs. PpR24b7	108.2	Yes	**	0.0099
PpR24a2 vs. PpR24b8	106.8	Yes	*	0.0124
PpR24a2 vs. PpR24b9	26.17	No	ns	>0.9999
PpR24a2 vs. PpR24b10	61.06	No	ns	>0.9999
PpR24a3 vs. PpR24a4	-4.5	No	ns	>0.9999
PpR24a3 vs. PpR24a5	-13.11	No	ns	>0.9999
PpR24a3 vs. PpR24a6	-4.778	No	ns	>0.9999
PpR24a3 vs. PpR24a7	0.9444	No	ns	>0.9999

PpR24a3 vs. PpR24a8	20	No	ns	>0.9999
PpR24a3 vs. PpR24a9	4.333	No	ns	>0.9999
PpR24a3 vs. PpR24a10	9.188	No	ns	>0.9999
PpR24a3 vs. PpR24b1	96.33	No	ns	0.0625
PpR24a3 vs. PpR24b2	102.5	Yes	*	0.0246
PpR24a3 vs. PpR24b3	76.17	No	ns	0.9199
PpR24a3 vs. PpR24b4	79.89	No	ns	0.5834
PpR24a3 vs. PpR24b5	35.28	No	ns	>0.9999
PpR24a3 vs. PpR24b6	85.39	No	ns	0.2877
PpR24a3 vs. PpR24b7	86.5	No	ns	0.2482
PpR24a3 vs. PpR24b8	85.11	No	ns	0.2985
PpR24a3 vs. PpR24b9	4.444	No	ns	>0.9999
PpR24a3 vs. PpR24b10	39.33	No	ns	>0.9999
PpR24a4 vs. PpR24a5	-8.611	No	ns	>0.9999
PpR24a4 vs. PpR24a6	-0.2778	No	ns	>0.9999
PpR24a4 vs. PpR24a7	5.444	No	ns	>0.9999
PpR24a4 vs. PpR24a8	24.5	No	ns	>0.9999
PpR24a4 vs. PpR24a9	8.833	No	ns	>0.9999
PpR24a4 vs. PpR24a10	13.69	No	ns	>0.9999
PpR24a4 vs. PpR24b1	100.8	Yes	*	0.0318
PpR24a4 vs. PpR24b2	107	Yes	*	0.0121
PpR24a4 vs. PpR24b3	80.67	No	ns	0.5292
PpR24a4 vs. PpR24b4	84.39	No	ns	0.3282
PpR24a4 vs. PpR24b5	39.78	No	ns	>0.9999
PpR24a4 vs. PpR24b6	89.89	No	ns	0.1566
PpR24a4 vs. PpR24b7	91	No	ns	0.1342
PpR24a4 vs. PpR24b8	89.61	No	ns	0.1627
PpR24a4 vs. PpR24b9	8.944	No	ns	>0.9999
PpR24a4 vs. PpR24b10	43.83	No	ns	>0.9999
PpR24a5 vs. PpR24a6	8.333	No	ns	>0.9999
PpR24a5 vs. PpR24a7	14.06	No	ns	>0.9999
PpR24a5 vs. PpR24a8	33.11	No	ns	>0.9999
PpR24a5 vs. PpR24a9	17.44	No	ns	>0.9999
PpR24a5 vs. PpR24a10	22.3	No	ns	>0.9999

PpR24a5 vs. PpR24b1	109.4	Yes	**	0.0081
PpR24a5 vs. PpR24b2	115.6	Yes	**	0.0029
PpR24a5 vs. PpR24b3	89.28	No	ns	0.1704
PpR24a5 vs. PpR24b4	93	No	ns	0.1012
PpR24a5 vs. PpR24b5	48.39	No	ns	>0.9999
PpR24a5 vs. PpR24b6	98.5	Yes	*	0.0453
PpR24a5 vs. PpR24b7	99.61	Yes	*	0.0383
PpR24a5 vs. PpR24b8	98.22	Yes	*	0.0472
PpR24a5 vs. PpR24b9	17.56	No	ns	>0.9999
PpR24a5 vs. PpR24b10	52.44	No	ns	>0.9999
PpR24a6 vs. PpR24a7	5.722	No	ns	>0.9999
PpR24a6 vs. PpR24a8	24.78	No	ns	>0.9999
PpR24a6 vs. PpR24a9	9.111	No	ns	>0.9999
PpR24a6 vs. PpR24a10	13.97	No	ns	>0.9999
PpR24a6 vs. PpR24b1	101.1	Yes	*	0.0305
PpR24a6 vs. PpR24b2	107.3	Yes	*	0.0115
PpR24a6 vs. PpR24b3	80.94	No	ns	0.511
PpR24a6 vs. PpR24b4	84.67	No	ns	0.3165
PpR24a6 vs. PpR24b5	40.06	No	ns	>0.9999
PpR24a6 vs. PpR24b6	90.17	No	ns	0.1507
PpR24a6 vs. PpR24b7	91.28	No	ns	0.1291
PpR24a6 vs. PpR24b8	89.89	No	ns	0.1566
PpR24a6 vs. PpR24b9	9.222	No	ns	>0.9999
PpR24a6 vs. PpR24b10	44.11	No	ns	>0.9999
PpR24a7 vs. PpR24a8	19.06	No	ns	>0.9999
PpR24a7 vs. PpR24a9	3.389	No	ns	>0.9999
PpR24a7 vs. PpR24a10	8.243	No	ns	>0.9999
PpR24a7 vs. PpR24b1	95.39	No	ns	0.0718
PpR24a7 vs. PpR24b2	101.6	Yes	*	0.0285
PpR24a7 vs. PpR24b3	75.22	No	ns	>0.9999
PpR24a7 vs. PpR24b4	78.94	No	ns	0.656
PpR24a7 vs. PpR24b5	34.33	No	ns	>0.9999
PpR24a7 vs. PpR24b6	84.44	No	ns	0.3258
PpR24a7 vs. PpR24b7	85.56	No	ns	0.2815

PpR24a7 vs. PpR24b8	84.17	No	ns	0.3379
PpR24a7 vs. PpR24b9	3.5	No	ns	>0.9999
PpR24a7 vs. PpR24b10	38.39	No	ns	>0.9999
PpR24a8 vs. PpR24a9	-15.67	No	ns	>0.9999
PpR24a8 vs. PpR24a10	-10.81	No	ns	>0.9999
PpR24a8 vs. PpR24b1	76.33	No	ns	>0.9999
PpR24a8 vs. PpR24b2	82.5	No	ns	0.8149
PpR24a8 vs. PpR24b3	56.17	No	ns	>0.9999
PpR24a8 vs. PpR24b4	59.89	No	ns	>0.9999
PpR24a8 vs. PpR24b5	15.28	No	ns	>0.9999
PpR24a8 vs. PpR24b6	65.39	No	ns	>0.9999
PpR24a8 vs. PpR24b7	66.5	No	ns	>0.9999
PpR24a8 vs. PpR24b8	65.11	No	ns	>0.9999
PpR24a8 vs. PpR24b9	-15.56	No	ns	>0.9999
PpR24a8 vs. PpR24b10	19.33	No	ns	>0.9999
PpR24a9 vs. PpR24a10	4.854	No	ns	>0.9999
PpR24a9 vs. PpR24b1	92	No	ns	0.1166
PpR24a9 vs. PpR24b2	98.17	Yes	*	0.0476
PpR24a9 vs. PpR24b3	71.83	No	ns	>0.9999
PpR24a9 vs. PpR24b4	75.56	No	ns	0.9896
PpR24a9 vs. PpR24b5	30.94	No	ns	>0.9999
PpR24a9 vs. PpR24b6	81.06	No	ns	0.5038
PpR24a9 vs. PpR24b7	82.17	No	ns	0.4375
PpR24a9 vs. PpR24b8	80.78	No	ns	0.5218
PpR24a9 vs. PpR24b9	0.1111	No	ns	>0.9999
PpR24a9 vs. PpR24b10	35	No	ns	>0.9999
PpR24a10 vs. PpR24b1	87.15	No	ns	0.3216
PpR24a10 vs. PpR24b2	93.31	No	ns	0.1434
PpR24a10 vs. PpR24b3	66.98	No	ns	>0.9999
PpR24a10 vs. PpR24b4	70.7	No	ns	>0.9999
PpR24a10 vs. PpR24b5	26.09	No	ns	>0.9999
PpR24a10 vs. PpR24b6	76.2	No	ns	>0.9999
PpR24a10 vs. PpR24b7	77.31	No	ns	>0.9999
PpR24a10 vs. PpR24b8	75.92	No	ns	>0.9999

PpR24a10 vs. PpR24b9	-4.743	No	ns	>0.9999
PpR24a10 vs. PpR24b10	30.15	No	ns	>0.9999
PpR24b1 vs. PpR24b2	6.167	No	ns	>0.9999
PpR24b1 vs. PpR24b3	-20.17	No	ns	>0.9999
PpR24b1 vs. PpR24b4	-16.44	No	ns	>0.9999
PpR24b1 vs. PpR24b5	-61.06	No	ns	>0.9999
PpR24b1 vs. PpR24b6	-10.94	No	ns	>0.9999
PpR24b1 vs. PpR24b7	-9.833	No	ns	>0.9999
PpR24b1 vs. PpR24b8	-11.22	No	ns	>0.9999
PpR24b1 vs. PpR24b9	-91.89	No	ns	0.1185
PpR24b1 vs. PpR24b10	-57	No	ns	>0.9999
PpR24b2 vs. PpR24b3	-26.33	No	ns	>0.9999
PpR24b2 vs. PpR24b4	-22.61	No	ns	>0.9999
PpR24b2 vs. PpR24b5	-67.22	No	ns	>0.9999
PpR24b2 vs. PpR24b6	-17.11	No	ns	>0.9999
PpR24b2 vs. PpR24b7	-16	No	ns	>0.9999
PpR24b2 vs. PpR24b8	-17.39	No	ns	>0.9999
PpR24b2 vs. PpR24b9	-98.06	Yes	*	0.0484
PpR24b2 vs. PpR24b10	-63.17	No	ns	>0.9999
PpR24b3 vs. PpR24b4	3.722	No	ns	>0.9999
PpR24b3 vs. PpR24b5	-40.89	No	ns	>0.9999
PpR24b3 vs. PpR24b6	9.222	No	ns	>0.9999
PpR24b3 vs. PpR24b7	10.33	No	ns	>0.9999
PpR24b3 vs. PpR24b8	8.944	No	ns	>0.9999
PpR24b3 vs. PpR24b9	-71.72	No	ns	>0.9999
PpR24b3 vs. PpR24b10	-36.83	No	ns	>0.9999
PpR24b4 vs. PpR24b5	-44.61	No	ns	>0.9999
PpR24b4 vs. PpR24b6	5.5	No	ns	>0.9999
PpR24b4 vs. PpR24b7	6.611	No	ns	>0.9999
PpR24b4 vs. PpR24b8	5.222	No	ns	>0.9999
PpR24b4 vs. PpR24b9	-75.44	No	ns	>0.9999
PpR24b4 vs. PpR24b10	-40.56	No	ns	>0.9999
PpR24b5 vs. PpR24b6	50.11	No	ns	>0.9999
PpR24b5 vs. PpR24b7	51.22	No	ns	>0.9999

PpR24b5 vs. PpR24b8	49.83	No	ns	>0.9999
PpR24b5 vs. PpR24b9	-30.83	No	ns	>0.9999
PpR24b5 vs. PpR24b10	4.056	No	ns	>0.9999
PpR24b6 vs. PpR24b7	1.111	No	ns	>0.9999
PpR24b6 vs. PpR24b8	-0.2778	No	ns	>0.9999
PpR24b6 vs. PpR24b9	-80.94	No	ns	0.511
PpR24b6 vs. PpR24b10	-46.06	No	ns	>0.9999
PpR24b7 vs. PpR24b8	-1.389	No	ns	>0.9999
PpR24b7 vs. PpR24b9	-82.06	No	ns	0.4437
PpR24b7 vs. PpR24b10	-47.17	No	ns	>0.9999
PpR24b8 vs. PpR24b9	-80.67	No	ns	0.5292
PpR24b8 vs. PpR24b10	-45.78	No	ns	>0.9999
PpR24b9 vs. PpR24b10	34.89	No	ns	>0.9999

B.1.2 Trade-off in biofilm maximum deformation mass and attachment strength

Table B.2: Complete pairwise differences for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment for biofilm maximum deformation mass of wild-type and evolved isolates. Kruskal-Wallis test p-value = <0.0001.

Dunn's multiple comparisons test	Mean rank diff.	Sign.?	Summary	Adjusted P-Value
WT vs. PpR24a1	0	No	ns	0.0014
WT vs. PpR24a2	0	No	ns	0.0001
WT vs. PpR24a3	0	No	ns	0.0001
WT vs. PpR24a4	0	No	ns	0.0058
WT vs. PpR24a5	0	No	ns	0.0027
WT vs. PpR24a6	0	No	ns	0.0006
WT vs. PpR24a7	0	No	ns	0.0025
WT vs. PpR24a8	0	No	ns	0.0067
WT vs. PpR24a9	0	No	ns	0.2802
WT vs. PpR24a10	0	No	ns	0.0118
WT vs. PpR24b1	-90.33	Yes	***	0.0404
WT vs. PpR24b2	-56.78	No	ns	>0.9999
WT vs. PpR24b3	-39.11	No	ns	>0.9999
WT vs. PpR24b4	-86.33	Yes	**	>0.9999
WT vs. PpR24b5	-60.56	No	ns	>0.9999
WT vs. PpR24b6	-65.33	No	ns	0.9138
WT vs. PpR24b7	0	No	ns	>0.9999
WT vs. PpR24b8	-53.06	No	ns	>0.9999
WT vs. PpR24b9	0	No	ns	>0.9999
WT vs. PpR24b10	0	No	ns	0.012
PpR24a1 vs. PpR24a2	0	No	ns	>0.9999
PpR24a1 vs. PpR24a3	0	No	ns	>0.9999
PpR24a1 vs. PpR24a4	0	No	ns	>0.9999
PpR24a1 vs. PpR24a5	0	No	ns	>0.9999
PpR24a1 vs. PpR24a6	0	No	ns	>0.9999
PpR24a1 vs. PpR24a7	0	No	ns	>0.9999

PpR24a1 vs. PpR24a8	0	No	ns	>0.9999
PpR24a1 vs. PpR24a9	0	No	ns	>0.9999
PpR24a1 vs. PpR24a10	0	No	ns	>0.9999
PpR24a1 vs. PpR24b1	-90.33	Yes	***	>0.9999
PpR24a1 vs. PpR24b2	-56.78	No	ns	>0.9999
PpR24a1 vs. PpR24b3	-39.11	No	ns	0.0183
PpR24a1 vs. PpR24b4	-86.33	Yes	**	0.0067
PpR24a1 vs. PpR24b5	-60.56	No	ns	0.3354
PpR24a1 vs. PpR24b6	-65.33	No	ns	0.2044
PpR24a1 vs. PpR24b7	0	No	ns	>0.9999
PpR24a1 vs. PpR24b8	-53.06	No	ns	0.095
PpR24a1 vs. PpR24b9	0	No	ns	0.081
PpR24a1 vs. PpR24b10	0	No	ns	0.0988
PpR24a2 vs. PpR24a3	0	No	ns	>0.9999
PpR24a2 vs. PpR24a4	0	No	ns	>0.9999
PpR24a2 vs. PpR24a5	0	No	ns	>0.9999
PpR24a2 vs. PpR24a6	0	No	ns	>0.9999
PpR24a2 vs. PpR24a7	0	No	ns	>0.9999
PpR24a2 vs. PpR24a8	0	No	ns	>0.9999
PpR24a2 vs. PpR24a9	0	No	ns	>0.9999
PpR24a2 vs. PpR24a10	0	No	ns	>0.9999
PpR24a2 vs. PpR24b1	-90.33	Yes	***	>0.9999
PpR24a2 vs. PpR24b2	-56.78	No	ns	>0.9999
PpR24a2 vs. PpR24b3	-39.11	No	ns	>0.9999
PpR24a2 vs. PpR24b4	-86.33	Yes	**	0.0021
PpR24a2 vs. PpR24b5	-60.56	No	ns	0.0007
PpR24a2 vs. PpR24b6	-65.33	No	ns	0.0557
PpR24a2 vs. PpR24b7	0	No	ns	0.0318
PpR24a2 vs. PpR24b8	-53.06	No	ns	>0.9999
PpR24a2 vs. PpR24b9	0	No	ns	0.0134
PpR24a2 vs. PpR24b10	0	No	ns	0.0112
PpR24a3 vs. PpR24a4	0	No	ns	0.0141
PpR24a3 vs. PpR24a5	0	No	ns	>0.9999
PpR24a3 vs. PpR24a6	0	No	ns	>0.9999

PpR24a3 vs. PpR24a7	0	No	ns	>0.9999
PpR24a3 vs. PpR24a8	0	No	ns	>0.9999
PpR24a3 vs. PpR24a9	0	No	ns	>0.9999
PpR24a3 vs. PpR24a10	0	No	ns	>0.9999
PpR24a3 vs. PpR24b1	-90.33	Yes	***	>0.9999
PpR24a3 vs. PpR24b2	-56.78	No	ns	>0.9999
PpR24a3 vs. PpR24b3	-39.11	No	ns	>0.9999
PpR24a3 vs. PpR24b4	-86.33	Yes	**	>0.9999
PpR24a3 vs. PpR24b5	-60.56	No	ns	0.0019
PpR24a3 vs. PpR24b6	-65.33	No	ns	0.0006
PpR24a3 vs. PpR24b7	0	No	ns	0.0496
PpR24a3 vs. PpR24b8	-53.06	No	ns	0.0282
PpR24a3 vs. PpR24b9	0	No	ns	>0.9999
PpR24a3 vs. PpR24b10	0	No	ns	0.0119
PpR24a4 vs. PpR24a5	0	No	ns	0.0099
PpR24a4 vs. PpR24a6	0	No	ns	0.0124
PpR24a4 vs. PpR24a7	0	No	ns	>0.9999
PpR24a4 vs. PpR24a8	0	No	ns	>0.9999
PpR24a4 vs. PpR24a9	0	No	ns	>0.9999
PpR24a4 vs. PpR24a10	0	No	ns	>0.9999
PpR24a4 vs. PpR24b1	-90.33	Yes	***	>0.9999
PpR24a4 vs. PpR24b2	-56.78	No	ns	>0.9999
PpR24a4 vs. PpR24b3	-39.11	No	ns	>0.9999
PpR24a4 vs. PpR24b4	-86.33	Yes	**	>0.9999
PpR24a4 vs. PpR24b5	-60.56	No	ns	>0.9999
PpR24a4 vs. PpR24b6	-65.33	No	ns	0.0625
PpR24a4 vs. PpR24b7	0	No	ns	0.0246
PpR24a4 vs. PpR24b8	-53.06	No	ns	0.9199
PpR24a4 vs. PpR24b9	0	No	ns	0.5834
PpR24a4 vs. PpR24b10	0	No	ns	>0.9999
PpR24a5 vs. PpR24a6	0	No	ns	0.2877
PpR24a5 vs. PpR24a7	0	No	ns	0.2482
PpR24a5 vs. PpR24a8	0	No	ns	0.2985
PpR24a5 vs. PpR24a9	0	No	ns	>0.9999

PpR24a5 vs. PpR24a10	0	No	ns	>0.9999
PpR24a5 vs. PpR24b1	-90.33	Yes	***	>0.9999
PpR24a5 vs. PpR24b2	-56.78	No	ns	>0.9999
PpR24a5 vs. PpR24b3	-39.11	No	ns	>0.9999
PpR24a5 vs. PpR24b4	-86.33	Yes	**	>0.9999
PpR24a5 vs. PpR24b5	-60.56	No	ns	>0.9999
PpR24a5 vs. PpR24b6	-65.33	No	ns	>0.9999
PpR24a5 vs. PpR24b7	0	No	ns	0.0318
PpR24a5 vs. PpR24b8	-53.06	No	ns	0.0121
PpR24a5 vs. PpR24b9	0	No	ns	0.5292
PpR24a5 vs. PpR24b10	0	No	ns	0.3282
PpR24a6 vs. PpR24a7	0	No	ns	>0.9999
PpR24a6 vs. PpR24a8	0	No	ns	0.1566
PpR24a6 vs. PpR24a9	0	No	ns	0.1342
PpR24a6 vs. PpR24a10	0	No	ns	0.1627
PpR24a6 vs. PpR24b1	-90.33	Yes	***	>0.9999
PpR24a6 vs. PpR24b2	-56.78	No	ns	>0.9999
PpR24a6 vs. PpR24b3	-39.11	No	ns	>0.9999
PpR24a6 vs. PpR24b4	-86.33	Yes	**	>0.9999
PpR24a6 vs. PpR24b5	-60.56	No	ns	>0.9999
PpR24a6 vs. PpR24b6	-65.33	No	ns	>0.9999
PpR24a6 vs. PpR24b7	0	No	ns	>0.9999
PpR24a6 vs. PpR24b8	-53.06	No	ns	0.0081
PpR24a6 vs. PpR24b9	0	No	ns	0.0029
PpR24a6 vs. PpR24b10	0	No	ns	0.1704
PpR24a7 vs. PpR24a8	0	No	ns	0.1012
PpR24a7 vs. PpR24a9	0	No	ns	>0.9999
PpR24a7 vs. PpR24a10	0	No	ns	0.0453
PpR24a7 vs. PpR24b1	-90.33	Yes	***	0.0383
PpR24a7 vs. PpR24b2	-56.78	No	ns	0.0472
PpR24a7 vs. PpR24b3	-39.11	No	ns	>0.9999
PpR24a7 vs. PpR24b4	-86.33	Yes	**	>0.9999
PpR24a7 vs. PpR24b5	-60.56	No	ns	>0.9999
PpR24a7 vs. PpR24b6	-65.33	No	ns	>0.9999

PpR24a7 vs. PpR24b7	0	No	ns	>0.9999
PpR24a7 vs. PpR24b8	-53.06	No	ns	>0.9999
PpR24a7 vs. PpR24b9	0	No	ns	0.0305
PpR24a7 vs. PpR24b10	0	No	ns	0.0115
PpR24a8 vs. PpR24a9	0	No	ns	0.511
PpR24a8 vs. PpR24a10	0	No	ns	0.3165
PpR24a8 vs. PpR24b1	-90.33	Yes	***	>0.9999
PpR24a8 vs. PpR24b2	-56.78	No	ns	0.1507
PpR24a8 vs. PpR24b3	-39.11	No	ns	0.1291
PpR24a8 vs. PpR24b4	-86.33	Yes	**	0.1566
PpR24a8 vs. PpR24b5	-60.56	No	ns	>0.9999
PpR24a8 vs. PpR24b6	-65.33	No	ns	>0.9999
PpR24a8 vs. PpR24b7	0	No	ns	>0.9999
PpR24a8 vs. PpR24b8	-53.06	No	ns	>0.9999
PpR24a8 vs. PpR24b9	0	No	ns	>0.9999
PpR24a8 vs. PpR24b10	0	No	ns	0.0718
PpR24a9 vs. PpR24a10	0	No	ns	0.0285
PpR24a9 vs. PpR24b1	-90.33	Yes	***	>0.9999
PpR24a9 vs. PpR24b2	-56.78	No	ns	0.656
PpR24a9 vs. PpR24b3	-39.11	No	ns	>0.9999
PpR24a9 vs. PpR24b4	-86.33	Yes	**	0.3258
PpR24a9 vs. PpR24b5	-60.56	No	ns	0.2815
PpR24a9 vs. PpR24b6	-65.33	No	ns	0.3379
PpR24a9 vs. PpR24b7	0	No	ns	>0.9999
PpR24a9 vs. PpR24b8	-53.06	No	ns	>0.9999
PpR24a9 vs. PpR24b9	0	No	ns	>0.9999
PpR24a9 vs. PpR24b10	0	No	ns	>0.9999
PpR24a10 vs. PpR24b1	-90.33	Yes	***	>0.9999
PpR24a10 vs. PpR24b2	-56.78	No	ns	0.8149
PpR24a10 vs. PpR24b3	-39.11	No	ns	>0.9999
PpR24a10 vs. PpR24b4	-86.33	Yes	**	>0.9999
PpR24a10 vs. PpR24b5	-60.56	No	ns	>0.9999
PpR24a10 vs. PpR24b6	-65.33	No	ns	>0.9999
PpR24a10 vs. PpR24b7	0	No	ns	>0.9999

PpR24a10 vs. PpR24b8	-53.06	No	ns	>0.9999
PpR24a10 vs. PpR24b9	0	No	ns	>0.9999
PpR24a10 vs. PpR24b10	0	No	ns	>0.9999
PpR24b1 vs. PpR24b2	33.56	No	ns	>0.9999
PpR24b1 vs. PpR24b3	51.22	No	ns	0.1166
PpR24b1 vs. PpR24b4	4	No	ns	0.0476
PpR24b1 vs. PpR24b5	29.78	No	ns	>0.9999
PpR24b1 vs. PpR24b6	25	No	ns	0.9896
PpR24b1 vs. PpR24b7	90.33	Yes	***	>0.9999
PpR24b1 vs. PpR24b8	37.28	No	ns	0.5038
PpR24b1 vs. PpR24b9	90.33	Yes	***	0.4375
PpR24b1 vs. PpR24b10	90.33	Yes	***	0.5218
PpR24b2 vs. PpR24b3	17.67	No	ns	>0.9999
PpR24b2 vs. PpR24b4	-29.56	No	ns	>0.9999
PpR24b2 vs. PpR24b5	-3.778	No	ns	0.3216
PpR24b2 vs. PpR24b6	-8.556	No	ns	0.1434
PpR24b2 vs. PpR24b7	56.78	No	ns	>0.9999
PpR24b2 vs. PpR24b8	3.722	No	ns	>0.9999
PpR24b2 vs. PpR24b9	56.78	No	ns	>0.9999
PpR24b2 vs. PpR24b10	56.78	No	ns	>0.9999
PpR24b3 vs. PpR24b4	-47.22	No	ns	>0.9999
PpR24b3 vs. PpR24b5	-21.44	No	ns	>0.9999
PpR24b3 vs. PpR24b6	-26.22	No	ns	>0.9999
PpR24b3 vs. PpR24b7	39.11	No	ns	>0.9999
PpR24b3 vs. PpR24b8	-13.94	No	ns	>0.9999
PpR24b3 vs. PpR24b9	39.11	No	ns	>0.9999
PpR24b3 vs. PpR24b10	39.11	No	ns	>0.9999
PpR24b4 vs. PpR24b5	25.78	No	ns	>0.9999
PpR24b4 vs. PpR24b6	21	No	ns	>0.9999
PpR24b4 vs. PpR24b7	86.33	Yes	**	>0.9999
PpR24b4 vs. PpR24b8	33.28	No	ns	>0.9999
PpR24b4 vs. PpR24b9	86.33	Yes	**	0.1185
PpR24b4 vs. PpR24b10	86.33	Yes	**	>0.9999
PpR24b5 vs. PpR24b6	-4.778	No	ns	>0.9999

PpR24b5 vs. PpR24b7	60.56	No	ns	>0.9999
PpR24b5 vs. PpR24b8	7.5	No	ns	>0.9999
PpR24b5 vs. PpR24b9	60.56	No	ns	>0.9999
PpR24b5 vs. PpR24b10	60.56	No	ns	>0.9999
PpR24b6 vs. PpR24b7	65.33	No	ns	>0.9999
PpR24b6 vs. PpR24b8	12.28	No	ns	0.0484
PpR24b6 vs. PpR24b9	65.33	No	ns	>0.9999
PpR24b6 vs. PpR24b10	65.33	No	ns	>0.9999
PpR24b7 vs. PpR24b8	-53.06	No	ns	>0.9999
PpR24b7 vs. PpR24b9	0	No	ns	>0.9999
PpR24b7 vs. PpR24b10	0	No	ns	>0.9999
PpR24b8 vs. PpR24b9	53.06	No	ns	>0.9999
PpR24b8 vs. PpR24b10	53.06	No	ns	>0.9999
PpR24b9 vs. PpR24b10	0	No	ns	>0.9999
PpR24b4 vs. PpR24b5	-44.61	No	ns	>0.9999
PpR24b4 vs. PpR24b6	5.5	No	ns	>0.9999
PpR24b4 vs. PpR24b7	6.611	No	ns	>0.9999
PpR24b4 vs. PpR24b8	5.222	No	ns	>0.9999
PpR24b4 vs. PpR24b9	-75.44	No	ns	>0.9999
PpR24b4 vs. PpR24b10	-40.56	No	ns	>0.9999
PpR24b5 vs. PpR24b6	50.11	No	ns	>0.9999
PpR24b5 vs. PpR24b7	51.22	No	ns	>0.9999
PpR24b5 vs. PpR24b8	49.83	No	ns	>0.9999
PpR24b5 vs. PpR24b9	-30.83	No	ns	>0.9999
PpR24b5 vs. PpR24b10	4.056	No	ns	>0.9999
PpR24b6 vs. PpR24b7	1.111	No	ns	>0.9999
PpR24b6 vs. PpR24b8	-0.2778	No	ns	>0.9999
PpR24b6 vs. PpR24b9	-80.94	No	ns	0.511
PpR24b6 vs. PpR24b10	-46.06	No	ns	>0.9999
PpR24b7 vs. PpR24b8	-1.389	No	ns	>0.9999
PpR24b7 vs. PpR24b9	-82.06	No	ns	0.4437
PpR24b7 vs. PpR24b10	-47.17	No	ns	>0.9999
PpR24b8 vs. PpR24b9	-80.67	No	ns	0.5292
PpR24b8 vs. PpR24b10	-45.78	No	ns	>0.9999

PpR24b9 vs. PpR24b10	34.89	No	ns	>0.9999

Table B.3: Complete pairwise differences for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment of biofilm attachment strength between wild-type and evolved isolates. Kruskal-Wallis test p-value = <0.0001.

Dunn's multiple comparisons test	**Mean rank diff.**	**Sign.?**	**Summary**	**Adjusted P-Value**
WT vs. PpR24a1	34.94	No	ns	>0.9999
WT vs. PpR24a2	31.44	No	ns	>0.9999
WT vs. PpR24a3	3.778	No	ns	>0.9999
WT vs. PpR24a4	50.28	No	ns	>0.9999
WT vs. PpR24a5	12.17	No	ns	>0.9999
WT vs. PpR24a6	10.89	No	ns	>0.9999
WT vs. PpR24a7	46.06	No	ns	>0.9999
WT vs. PpR24a8	27.78	No	ns	>0.9999
WT vs. PpR24a9	26.72	No	ns	>0.9999
WT vs. PpR24a10	23.28	No	ns	>0.9999
WT vs. PpR24b1	-69.33	No	ns	>0.9999
WT vs. PpR24b2	-26.44	No	ns	>0.9999
WT vs. PpR24b3	-32.11	No	ns	>0.9999
WT vs. PpR24b4	-31.39	No	ns	>0.9999
WT vs. PpR24b5	40.72	No	ns	>0.9999
WT vs. PpR24b6	-55.83	No	ns	>0.9999
WT vs. PpR24b7	-36.67	No	ns	>0.9999
WT vs. PpR24b8	-58.39	No	ns	>0.9999
WT vs. PpR24b9	27.67	No	ns	>0.9999
WT vs. PpR24b10	-39.56	No	ns	>0.9999
PpR24a1 vs. PpR24a2	-3.5	No	ns	>0.9999
PpR24a1 vs. PpR24a3	-31.17	No	ns	>0.9999
PpR24a1 vs. PpR24a4	15.33	No	ns	>0.9999
PpR24a1 vs. PpR24a5	-22.78	No	ns	>0.9999
PpR24a1 vs. PpR24a6	-24.06	No	ns	>0.9999
PpR24a1 vs. PpR24a7	11.11	No	ns	>0.9999
PpR24a1 vs. PpR24a8	-7.167	No	ns	>0.9999
PpR24a1 vs. PpR24a9	-8.222	No	ns	>0.9999
PpR24a1 vs. PpR24a10	-11.67	No	ns	>0.9999

PpR24a1 vs. PpR24b1	-104.3	Yes	*	0.011
PpR24a1 vs. PpR24b2	-61.39	No	ns	>0.9999
PpR24a1 vs. PpR24b3	-67.06	No	ns	>0.9999
PpR24a1 vs. PpR24b4	-66.33	No	ns	>0.9999
PpR24a1 vs. PpR24b5	5.778	No	ns	>0.9999
PpR24a1 vs. PpR24b6	-90.78	No	ns	0.0905
PpR24a1 vs. PpR24b7	-71.61	No	ns	>0.9999
PpR24a1 vs. PpR24b8	-93.33	No	ns	0.062
PpR24a1 vs. PpR24b9	-7.278	No	ns	>0.9999
PpR24a1 vs. PpR24b10	-74.5	No	ns	0.8115
PpR24a2 vs. PpR24a3	-27.67	No	ns	>0.9999
PpR24a2 vs. PpR24a4	18.83	No	ns	>0.9999
PpR24a2 vs. PpR24a5	-19.28	No	ns	>0.9999
PpR24a2 vs. PpR24a6	-20.56	No	ns	>0.9999
PpR24a2 vs. PpR24a7	14.61	No	ns	>0.9999
PpR24a2 vs. PpR24a8	-3.667	No	ns	>0.9999
PpR24a2 vs. PpR24a9	-4.722	No	ns	>0.9999
PpR24a2 vs. PpR24a10	-8.167	No	ns	>0.9999
PpR24a2 vs. PpR24b1	-100.8	Yes	*	0.0195
PpR24a2 vs. PpR24b2	-57.89	No	ns	>0.9999
PpR24a2 vs. PpR24b3	-63.56	No	ns	>0.9999
PpR24a2 vs. PpR24b4	-62.83	No	ns	>0.9999
PpR24a2 vs. PpR24b5	9.278	No	ns	>0.9999
PpR24a2 vs. PpR24b6	-87.28	No	ns	0.1497
PpR24a2 vs. PpR24b7	-68.11	No	ns	>0.9999
PpR24a2 vs. PpR24b8	-89.83	No	ns	0.1039
PpR24a2 vs. PpR24b9	-3.778	No	ns	>0.9999
PpR24a2 vs. PpR24b10	-71	No	ns	>0.9999
PpR24a3 vs. PpR24a4	46.5	No	ns	>0.9999
PpR24a3 vs. PpR24a5	8.389	No	ns	>0.9999
PpR24a3 vs. PpR24a6	7.111	No	ns	>0.9999
PpR24a3 vs. PpR24a7	42.28	No	ns	>0.9999
PpR24a3 vs. PpR24a8	24	No	ns	>0.9999
PpR24a3 vs. PpR24a9	22.94	No	ns	>0.9999

PpR24a3 vs. PpR24a10	19.5	No	ns	>0.9999
PpR24a3 vs. PpR24b1	-73.11	No	ns	0.9618
PpR24a3 vs. PpR24b2	-30.22	No	ns	>0.9999
PpR24a3 vs. PpR24b3	-35.89	No	ns	>0.9999
PpR24a3 vs. PpR24b4	-35.17	No	ns	>0.9999
PpR24a3 vs. PpR24b5	36.94	No	ns	>0.9999
PpR24a3 vs. PpR24b6	-59.61	No	ns	>0.9999
PpR24a3 vs. PpR24b7	-40.44	No	ns	>0.9999
PpR24a3 vs. PpR24b8	-62.17	No	ns	>0.9999
PpR24a3 vs. PpR24b9	23.89	No	ns	>0.9999
PpR24a3 vs. PpR24b10	-43.33	No	ns	>0.9999
PpR24a4 vs. PpR24a5	-38.11	No	ns	>0.9999
PpR24a4 vs. PpR24a6	-39.39	No	ns	>0.9999
PpR24a4 vs. PpR24a7	-4.222	No	ns	>0.9999
PpR24a4 vs. PpR24a8	-22.5	No	ns	>0.9999
PpR24a4 vs. PpR24a9	-23.56	No	ns	>0.9999
PpR24a4 vs. PpR24a10	-27	No	ns	>0.9999
PpR24a4 vs. PpR24b1	-119.6	Yes	***	0.0007
PpR24a4 vs. PpR24b2	-76.72	No	ns	0.6148
PpR24a4 vs. PpR24b3	-82.39	No	ns	0.2937
PpR24a4 vs. PpR24b4	-81.67	No	ns	0.3235
PpR24a4 vs. PpR24b5	-9.556	No	ns	>0.9999
PpR24a4 vs. PpR24b6	-106.1	Yes	**	0.0081
PpR24a4 vs. PpR24b7	-86.94	No	ns	0.1569
PpR24a4 vs. PpR24b8	-108.7	Yes	**	0.0053
PpR24a4 vs. PpR24b9	-22.61	No	ns	>0.9999
PpR24a4 vs. PpR24b10	-89.83	No	ns	0.1039
PpR24a5 vs. PpR24a6	-1.278	No	ns	>0.9999
PpR24a5 vs. PpR24a7	33.89	No	ns	>0.9999
PpR24a5 vs. PpR24a8	15.61	No	ns	>0.9999
PpR24a5 vs. PpR24a9	14.56	No	ns	>0.9999
PpR24a5 vs. PpR24a10	11.11	No	ns	>0.9999
PpR24a5 vs. PpR24b1	-81.5	No	ns	0.3308
PpR24a5 vs. PpR24b2	-38.61	No	ns	>0.9999

PpR24a5 vs. PpR24b3	-44.28	No	ns	>0.9999
PpR24a5 vs. PpR24b4	-43.56	No	ns	>0.9999
PpR24a5 vs. PpR24b5	28.56	No	ns	>0.9999
PpR24a5 vs. PpR24b6	-68	No	ns	>0.9999
PpR24a5 vs. PpR24b7	-48.83	No	ns	>0.9999
PpR24a5 vs. PpR24b8	-70.56	No	ns	>0.9999
PpR24a5 vs. PpR24b9	15.5	No	ns	>0.9999
PpR24a5 vs. PpR24b10	-51.72	No	ns	>0.9999
PpR24a6 vs. PpR24a7	35.17	No	ns	>0.9999
PpR24a6 vs. PpR24a8	16.89	No	ns	>0.9999
PpR24a6 vs. PpR24a9	15.83	No	ns	>0.9999
PpR24a6 vs. PpR24a10	12.39	No	ns	>0.9999
PpR24a6 vs. PpR24b1	-80.22	No	ns	0.3916
PpR24a6 vs. PpR24b2	-37.33	No	ns	>0.9999
PpR24a6 vs. PpR24b3	-43	No	ns	>0.9999
PpR24a6 vs. PpR24b4	-42.28	No	ns	>0.9999
PpR24a6 vs. PpR24b5	29.83	No	ns	>0.9999
PpR24a6 vs. PpR24b6	-66.72	No	ns	>0.9999
PpR24a6 vs. PpR24b7	-47.56	No	ns	>0.9999
PpR24a6 vs. PpR24b8	-69.28	No	ns	>0.9999
PpR24a6 vs. PpR24b9	16.78	No	ns	>0.9999
PpR24a6 vs. PpR24b10	-50.44	No	ns	>0.9999
PpR24a7 vs. PpR24a8	-18.28	No	ns	>0.9999
PpR24a7 vs. PpR24a9	-19.33	No	ns	>0.9999
PpR24a7 vs. PpR24a10	-22.78	No	ns	>0.9999
PpR24a7 vs. PpR24b1	-115.4	Yes	**	0.0016
PpR24a7 vs. PpR24b2	-72.5	No	ns	>0.9999
PpR24a7 vs. PpR24b3	-78.17	No	ns	0.5115
PpR24a7 vs. PpR24b4	-77.44	No	ns	0.561
PpR24a7 vs. PpR24b5	-5.333	No	ns	>0.9999
PpR24a7 vs. PpR24b6	-101.9	Yes	*	0.0163
PpR24a7 vs. PpR24b7	-82.72	No	ns	0.2808
PpR24a7 vs. PpR24b8	-104.4	Yes	*	0.0107
PpR24a7 vs. PpR24b9	-18.39	No	ns	>0.9999

PpR24a7 vs. PpR24b10	-85.61	No	ns	0.1891
PpR24a8 vs. PpR24a9	-1.056	No	ns	>0.9999
PpR24a8 vs. PpR24a10	-4.5	No	ns	>0.9999
PpR24a8 vs. PpR24b1	-97.11	Yes	*	0.0349
PpR24a8 vs. PpR24b2	-54.22	No	ns	>0.9999
PpR24a8 vs. PpR24b3	-59.89	No	ns	>0.9999
PpR24a8 vs. PpR24b4	-59.17	No	ns	>0.9999
PpR24a8 vs. PpR24b5	12.94	No	ns	>0.9999
PpR24a8 vs. PpR24b6	-83.61	No	ns	0.2489
PpR24a8 vs. PpR24b7	-64.44	No	ns	>0.9999
PpR24a8 vs. PpR24b8	-86.17	No	ns	0.175
PpR24a8 vs. PpR24b9	-0.1111	No	ns	>0.9999
PpR24a8 vs. PpR24b10	-67.33	No	ns	>0.9999
PpR24a9 vs. PpR24a10	-3.444	No	ns	>0.9999
PpR24a9 vs. PpR24b1	-96.06	Yes	*	0.041
PpR24a9 vs. PpR24b2	-53.17	No	ns	>0.9999
PpR24a9 vs. PpR24b3	-58.83	No	ns	>0.9999
PpR24a9 vs. PpR24b4	-58.11	No	ns	>0.9999
PpR24a9 vs. PpR24b5	14	No	ns	>0.9999
PpR24a9 vs. PpR24b6	-82.56	No	ns	0.2872
PpR24a9 vs. PpR24b7	-63.39	No	ns	>0.9999
PpR24a9 vs. PpR24b8	-85.11	No	ns	0.2027
PpR24a9 vs. PpR24b9	0.9444	No	ns	>0.9999
PpR24a9 vs. PpR24b10	-66.28	No	ns	>0.9999
PpR24a10 vs. PpR24b1	-92.61	No	ns	0.0691
PpR24a10 vs. PpR24b2	-49.72	No	ns	>0.9999
PpR24a10 vs. PpR24b3	-55.39	No	ns	>0.9999
PpR24a10 vs. PpR24b4	-54.67	No	ns	>0.9999
PpR24a10 vs. PpR24b5	17.44	No	ns	>0.9999
PpR24a10 vs. PpR24b6	-79.11	No	ns	0.4528
PpR24a10 vs. PpR24b7	-59.94	No	ns	>0.9999
PpR24a10 vs. PpR24b8	-81.67	No	ns	0.3235
PpR24a10 vs. PpR24b9	4.389	No	ns	>0.9999
PpR24a10 vs. PpR24b10	-62.83	No	ns	>0.9999

PpR24b1 vs. PpR24b2	42.89	No	ns	>0.9999
PpR24b1 vs. PpR24b3	37.22	No	ns	>0.9999
PpR24b1 vs. PpR24b4	37.94	No	ns	>0.9999
PpR24b1 vs. PpR24b5	110.1	Yes	**	0.0041
PpR24b1 vs. PpR24b6	13.5	No	ns	>0.9999
PpR24b1 vs. PpR24b7	32.67	No	ns	>0.9999
PpR24b1 vs. PpR24b8	10.94	No	ns	>0.9999
PpR24b1 vs. PpR24b9	97	Yes	*	0.0355
PpR24b1 vs. PpR24b10	29.78	No	ns	>0.9999
PpR24b2 vs. PpR24b3	-5.667	No	ns	>0.9999
PpR24b2 vs. PpR24b4	-4.944	No	ns	>0.9999
PpR24b2 vs. PpR24b5	67.17	No	ns	>0.9999
PpR24b2 vs. PpR24b6	-29.39	No	ns	>0.9999
PpR24b2 vs. PpR24b7	-10.22	No	ns	>0.9999
PpR24b2 vs. PpR24b8	-31.94	No	ns	>0.9999
PpR24b2 vs. PpR24b9	54.11	No	ns	>0.9999
PpR24b2 vs. PpR24b10	-13.11	No	ns	>0.9999
PpR24b3 vs. PpR24b4	0.7222	No	ns	>0.9999
PpR24b3 vs. PpR24b5	72.83	No	ns	0.9947
PpR24b3 vs. PpR24b6	-23.72	No	ns	>0.9999
PpR24b3 vs. PpR24b7	-4.556	No	ns	>0.9999
PpR24b3 vs. PpR24b8	-26.28	No	ns	>0.9999
PpR24b3 vs. PpR24b9	59.78	No	ns	>0.9999
PpR24b3 vs. PpR24b10	-7.444	No	ns	>0.9999
PpR24b4 vs. PpR24b5	72.11	No	ns	>0.9999
PpR24b4 vs. PpR24b6	-24.44	No	ns	>0.9999
PpR24b4 vs. PpR24b7	-5.278	No	ns	>0.9999
PpR24b4 vs. PpR24b8	-27	No	ns	>0.9999
PpR24b4 vs. PpR24b9	59.06	No	ns	>0.9999
PpR24b4 vs. PpR24b10	-8.167	No	ns	>0.9999
PpR24b5 vs. PpR24b6	-96.56	Yes	*	0.038
PpR24b5 vs. PpR24b7	-77.39	No	ns	0.565
PpR24b5 vs. PpR24b8	-99.11	Yes	*	0.0255
PpR24b5 vs. PpR24b9	-13.06	No	ns	>0.9999

PpR24b5 vs. PpR24b10	-80.28	No	ns	0.3888
PpR24b6 vs. PpR24b7	19.17	No	ns	>0.9999
PpR24b6 vs. PpR24b8	-2.556	No	ns	>0.9999
PpR24b6 vs. PpR24b9	83.5	No	ns	0.2527
PpR24b6 vs. PpR24b10	16.28	No	ns	>0.9999
PpR24b7 vs. PpR24b8	-21.72	No	ns	>0.9999
PpR24b7 vs. PpR24b9	64.33	No	ns	>0.9999
PpR24b7 vs. PpR24b10	-2.889	No	ns	>0.9999
PpR24b8 vs. PpR24b9	86.06	No	ns	0.1777
PpR24b8 vs. PpR24b10	18.83	No	ns	>0.9999
PpR24b9 vs. PpR24b10	-67.22	No	ns	>0.9999
PpR24b9 vs. PpR24b10	-67.22	No	ns	>0.9999

B.1.3 Growth trade-offs

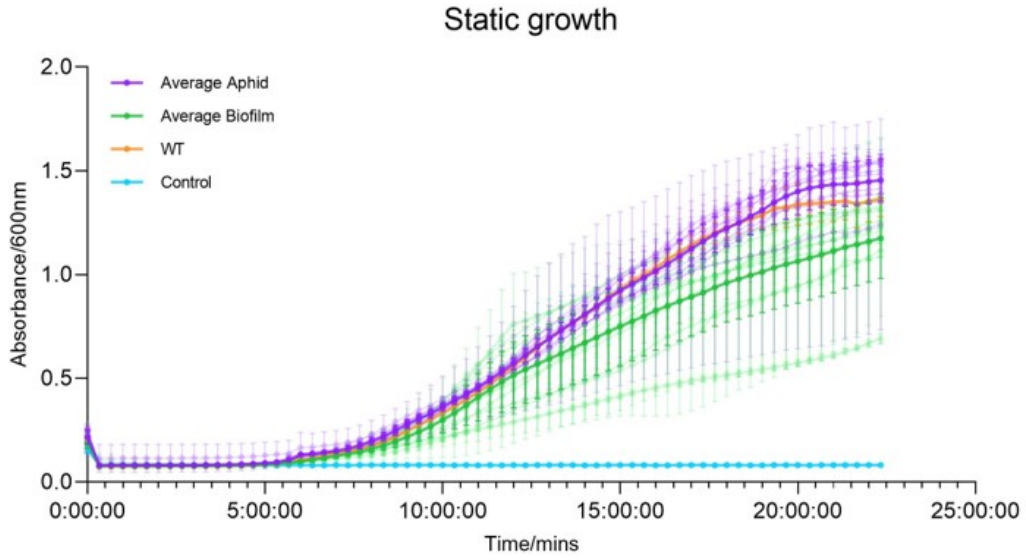

Figure B.1: Evolved isolate and wild-type PpR24 growth in static conditions (n=4)

Table B.4: Complete pairwise differences for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment for wild-type and evolved isolate growth in static conditions. Kruskal-Wallis test p-value = 0.0018.

Dunn's multiple comparisons test	**Mean rank diff.**	**Sign.?**	**Summary**	**Adjusted P-Value**
WT vs. Control	32.33	No	ns	>0.9999
WT vs. PpR24a1	-7.333	No	ns	>0.9999
WT vs. PpR24a2	-14.67	No	ns	>0.9999
WT vs. PpR24a3	-22.83	No	ns	>0.9999
WT vs. PpR24a4	-22.33	No	ns	>0.9999
WT vs. PpR24a5	-21	No	ns	>0.9999
WT vs. PpR24a6	-19.67	No	ns	>0.9999
WT vs. PpR24a7	-16	No	ns	>0.9999
WT vs. PpR24a8	-3	No	ns	>0.9999
WT vs. PpR24a9	0.6667	No	ns	>0.9999
WT vs. PpR24a10	-3.833	No	ns	>0.9999
WT vs. PpR24b1	13.5	No	ns	>0.9999

WT vs. PpR24b2	13.33	No	ns	>0.9999
WT vs. PpR24b3	11.67	No	ns	>0.9999
WT vs. PpR24b4	19.67	No	ns	>0.9999
WT vs. PpR24b5	15.33	No	ns	>0.9999
WT vs. PpR24b6	11	No	ns	>0.9999
WT vs. PpR24b7	6.333	No	ns	>0.9999
WT vs. PpR24b8	4.667	No	ns	>0.9999
WT vs. PpR24b9	27.33	No	ns	>0.9999
WT vs. PpR24b10	-6.833	No	ns	>0.9999
Control vs. PpR24a1	-39.67	No	ns	>0.9999
Control vs. PpR24a2	-47	No	ns	0.6262
Control vs. PpR24a3	-55.17	No	ns	0.0998
Control vs. PpR24a4	-54.67	No	ns	0.1125
Control vs. PpR24a5	-53.33	No	ns	0.1541
Control vs. PpR24a6	-52	No	ns	0.2096
Control vs. PpR24a7	-48.33	No	ns	0.4721
Control vs. PpR24a8	-35.33	No	ns	>0.9999
Control vs. PpR24a9	-31.67	No	ns	>0.9999
Control vs. PpR24a10	-36.17	No	ns	>0.9999
Control vs. PpR24b1	-18.83	No	ns	>0.9999
Control vs. PpR24b2	-19	No	ns	>0.9999
Control vs. PpR24b3	-20.67	No	ns	>0.9999
Control vs. PpR24b4	-12.67	No	ns	>0.9999
Control vs. PpR24b5	-17	No	ns	>0.9999
Control vs. PpR24b6	-21.33	No	ns	>0.9999
Control vs. PpR24b7	-26	No	ns	>0.9999
Control vs. PpR24b8	-27.67	No	ns	>0.9999
Control vs. PpR24b9	-5	No	ns	>0.9999
Control vs. PpR24b10	-39.17	No	ns	>0.9999
PpR24a1 vs. PpR24a2	-7.333	No	ns	>0.9999
PpR24a1 vs. PpR24a3	-15.5	No	ns	>0.9999
PpR24a1 vs. PpR24a4	-15	No	ns	>0.9999
PpR24a1 vs. PpR24a5	-13.67	No	ns	>0.9999
PpR24a1 vs. PpR24a6	-12.33	No	ns	>0.9999

PpR24a1 vs. PpR24a7	-8.667	No	ns	>0.9999
PpR24a1 vs. PpR24a8	4.333	No	ns	>0.9999
PpR24a1 vs. PpR24a9	8	No	ns	>0.9999
PpR24a1 vs. PpR24a10	3.5	No	ns	>0.9999
PpR24a1 vs. PpR24b1	20.83	No	ns	>0.9999
PpR24a1 vs. PpR24b2	20.67	No	ns	>0.9999
PpR24a1 vs. PpR24b3	19	No	ns	>0.9999
PpR24a1 vs. PpR24b4	27	No	ns	>0.9999
PpR24a1 vs. PpR24b5	22.67	No	ns	>0.9999
PpR24a1 vs. PpR24b6	18.33	No	ns	>0.9999
PpR24a1 vs. PpR24b7	13.67	No	ns	>0.9999
PpR24a1 vs. PpR24b8	12	No	ns	>0.9999
PpR24a1 vs. PpR24b9	34.67	No	ns	>0.9999
PpR24a1 vs. PpR24b10	0.5	No	ns	>0.9999
PpR24a2 vs. PpR24a3	-8.167	No	ns	>0.9999
PpR24a2 vs. PpR24a4	-7.667	No	ns	>0.9999
PpR24a2 vs. PpR24a5	-6.333	No	ns	>0.9999
PpR24a2 vs. PpR24a6	-5	No	ns	>0.9999
PpR24a2 vs. PpR24a7	-1.333	No	ns	>0.9999
PpR24a2 vs. PpR24a8	11.67	No	ns	>0.9999
PpR24a2 vs. PpR24a9	15.33	No	ns	>0.9999
PpR24a2 vs. PpR24a10	10.83	No	ns	>0.9999
PpR24a2 vs. PpR24b1	28.17	No	ns	>0.9999
PpR24a2 vs. PpR24b2	28	No	ns	>0.9999
PpR24a2 vs. PpR24b3	26.33	No	ns	>0.9999
PpR24a2 vs. PpR24b4	34.33	No	ns	>0.9999
PpR24a2 vs. PpR24b5	30	No	ns	>0.9999
PpR24a2 vs. PpR24b6	25.67	No	ns	>0.9999
PpR24a2 vs. PpR24b7	21	No	ns	>0.9999
PpR24a2 vs. PpR24b8	19.33	No	ns	>0.9999
PpR24a2 vs. PpR24b9	42	No	ns	>0.9999
PpR24a2 vs. PpR24b10	7.833	No	ns	>0.9999
PpR24a3 vs. PpR24a4	0.5	No	ns	>0.9999
PpR24a3 vs. PpR24a5	1.833	No	ns	>0.9999

PpR24a3 vs. PpR24a6	3.167	No	ns	>0.9999
PpR24a3 vs. PpR24a7	6.833	No	ns	>0.9999
PpR24a3 vs. PpR24a8	19.83	No	ns	>0.9999
PpR24a3 vs. PpR24a9	23.5	No	ns	>0.9999
PpR24a3 vs. PpR24a10	19	No	ns	>0.9999
PpR24a3 vs. PpR24b1	36.33	No	ns	>0.9999
PpR24a3 vs. PpR24b2	36.17	No	ns	>0.9999
PpR24a3 vs. PpR24b3	34.5	No	ns	>0.9999
PpR24a3 vs. PpR24b4	42.5	No	ns	>0.9999
PpR24a3 vs. PpR24b5	38.17	No	ns	>0.9999
PpR24a3 vs. PpR24b6	33.83	No	ns	>0.9999
PpR24a3 vs. PpR24b7	29.17	No	ns	>0.9999
PpR24a3 vs. PpR24b8	27.5	No	ns	>0.9999
PpR24a3 vs. PpR24b9	50.17	No	ns	0.3166
PpR24a3 vs. PpR24b10	16	No	ns	>0.9999
PpR24a4 vs. PpR24a5	1.333	No	ns	>0.9999
PpR24a4 vs. PpR24a6	2.667	No	ns	>0.9999
PpR24a4 vs. PpR24a7	6.333	No	ns	>0.9999
PpR24a4 vs. PpR24a8	19.33	No	ns	>0.9999
PpR24a4 vs. PpR24a9	23	No	ns	>0.9999
PpR24a4 vs. PpR24a10	18.5	No	ns	>0.9999
PpR24a4 vs. PpR24b1	35.83	No	ns	>0.9999
PpR24a4 vs. PpR24b2	35.67	No	ns	>0.9999
PpR24a4 vs. PpR24b3	34	No	ns	>0.9999
PpR24a4 vs. PpR24b4	42	No	ns	>0.9999
PpR24a4 vs. PpR24b5	37.67	No	ns	>0.9999
PpR24a4 vs. PpR24b6	33.33	No	ns	>0.9999
PpR24a4 vs. PpR24b7	28.67	No	ns	>0.9999
PpR24a4 vs. PpR24b8	27	No	ns	>0.9999
PpR24a4 vs. PpR24b9	49.67	No	ns	0.3535
PpR24a4 vs. PpR24b10	15.5	No	ns	>0.9999
PpR24a5 vs. PpR24a6	1.333	No	ns	>0.9999
PpR24a5 vs. PpR24a7	5	No	ns	>0.9999
PpR24a5 vs. PpR24a8	18	No	ns	>0.9999

PpR24a5 vs. PpR24a9	21.67	No	ns	>0.9999
PpR24a5 vs. PpR24a10	17.17	No	ns	>0.9999
PpR24a5 vs. PpR24b1	34.5	No	ns	>0.9999
PpR24a5 vs. PpR24b2	34.33	No	ns	>0.9999
PpR24a5 vs. PpR24b3	32.67	No	ns	>0.9999
PpR24a5 vs. PpR24b4	40.67	No	ns	>0.9999
PpR24a5 vs. PpR24b5	36.33	No	ns	>0.9999
PpR24a5 vs. PpR24b6	32	No	ns	>0.9999
PpR24a5 vs. PpR24b7	27.33	No	ns	>0.9999
PpR24a5 vs. PpR24b8	25.67	No	ns	>0.9999
PpR24a5 vs. PpR24b9	48.33	No	ns	0.4721
PpR24a5 vs. PpR24b10	14.17	No	ns	>0.9999
PpR24a6 vs. PpR24a7	3.667	No	ns	>0.9999
PpR24a6 vs. PpR24a8	16.67	No	ns	>0.9999
PpR24a6 vs. PpR24a9	20.33	No	ns	>0.9999
PpR24a6 vs. PpR24a10	15.83	No	ns	>0.9999
PpR24a6 vs. PpR24b1	33.17	No	ns	>0.9999
PpR24a6 vs. PpR24b2	33	No	ns	>0.9999
PpR24a6 vs. PpR24b3	31.33	No	ns	>0.9999
PpR24a6 vs. PpR24b4	39.33	No	ns	>0.9999
PpR24a6 vs. PpR24b5	35	No	ns	>0.9999
PpR24a6 vs. PpR24b6	30.67	No	ns	>0.9999
PpR24a6 vs. PpR24b7	26	No	ns	>0.9999
PpR24a6 vs. PpR24b8	24.33	No	ns	>0.9999
PpR24a6 vs. PpR24b9	47	No	ns	0.6262
PpR24a6 vs. PpR24b10	12.83	No	ns	>0.9999
PpR24a7 vs. PpR24a8	13	No	ns	>0.9999
PpR24a7 vs. PpR24a9	16.67	No	ns	>0.9999
PpR24a7 vs. PpR24a10	12.17	No	ns	>0.9999
PpR24a7 vs. PpR24b1	29.5	No	ns	>0.9999
PpR24a7 vs. PpR24b2	29.33	No	ns	>0.9999
PpR24a7 vs. PpR24b3	27.67	No	ns	>0.9999
PpR24a7 vs. PpR24b4	35.67	No	ns	>0.9999
PpR24a7 vs. PpR24b5	31.33	No	ns	>0.9999

PpR24a7 vs. PpR24b6	27	No	ns	>0.9999
PpR24a7 vs. PpR24b7	22.33	No	ns	>0.9999
PpR24a7 vs. PpR24b8	20.67	No	ns	>0.9999
PpR24a7 vs. PpR24b9	43.33	No	ns	>0.9999
PpR24a7 vs. PpR24b10	9.167	No	ns	>0.9999
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PpR24a8 vs. PpR24b3	14.67	No	ns	>0.9999
PpR24a8 vs. PpR24b4	22.67	No	ns	>0.9999
PpR24a8 vs. PpR24b5	18.33	No	ns	>0.9999
PpR24a8 vs. PpR24b6	14	No	ns	>0.9999
PpR24a8 vs. PpR24b7	9.333	No	ns	>0.9999
PpR24a8 vs. PpR24b8	7.667	No	ns	>0.9999
PpR24a8 vs. PpR24b9	30.33	No	ns	>0.9999
PpR24a8 vs. PpR24b10	-3.833	No	ns	>0.9999
PpR24a9 vs. PpR24a10	-4.5	No	ns	>0.9999
PpR24a9 vs. PpR24b1	12.83	No	ns	>0.9999
PpR24a9 vs. PpR24b2	12.67	No	ns	>0.9999
PpR24a9 vs. PpR24b3	11	No	ns	>0.9999
PpR24a9 vs. PpR24b4	19	No	ns	>0.9999
PpR24a9 vs. PpR24b5	14.67	No	ns	>0.9999
PpR24a9 vs. PpR24b6	10.33	No	ns	>0.9999
PpR24a9 vs. PpR24b7	5.667	No	ns	>0.9999
PpR24a9 vs. PpR24b8	4	No	ns	>0.9999
PpR24a9 vs. PpR24b9	26.67	No	ns	>0.9999
PpR24a9 vs. PpR24b10	-7.5	No	ns	>0.9999
PpR24a10 vs. PpR24b1	17.33	No	ns	>0.9999
PpR24a10 vs. PpR24b2	17.17	No	ns	>0.9999
PpR24a10 vs. PpR24b3	15.5	No	ns	>0.9999
PpR24a10 vs. PpR24b4	23.5	No	ns	>0.9999
PpR24a10 vs. PpR24b5	19.17	No	ns	>0.9999
PpR24a10 vs. PpR24b6	14.83	No	ns	>0.9999

PpR24a10 vs. PpR24b7	10.17	No	ns	>0.9999
PpR24a10 vs. PpR24b8	8.5	No	ns	>0.9999
PpR24a10 vs. PpR24b9	31.17	No	ns	>0.9999
PpR24a10 vs. PpR24b10	-3	No	ns	>0.9999
PpR24b1 vs. PpR24b2	-0.1667	No	ns	>0.9999
PpR24b1 vs. PpR24b3	-1.833	No	ns	>0.9999
PpR24b1 vs. PpR24b4	6.167	No	ns	>0.9999
PpR24b1 vs. PpR24b5	1.833	No	ns	>0.9999
PpR24b1 vs. PpR24b6	-2.5	No	ns	>0.9999
PpR24b1 vs. PpR24b7	-7.167	No	ns	>0.9999
PpR24b1 vs. PpR24b8	-8.833	No	ns	>0.9999
PpR24b1 vs. PpR24b9	13.83	No	ns	>0.9999
PpR24b1 vs. PpR24b10	-20.33	No	ns	>0.9999
PpR24b2 vs. PpR24b3	-1.667	No	ns	>0.9999
PpR24b2 vs. PpR24b4	6.333	No	ns	>0.9999
PpR24b2 vs. PpR24b5	2	No	ns	>0.9999
PpR24b2 vs. PpR24b6	-2.333	No	ns	>0.9999
PpR24b2 vs. PpR24b7	-7	No	ns	>0.9999
PpR24b2 vs. PpR24b8	-8.667	No	ns	>0.9999
PpR24b2 vs. PpR24b9	14	No	ns	>0.9999
PpR24b2 vs. PpR24b10	-20.17	No	ns	>0.9999
PpR24b3 vs. PpR24b4	8	No	ns	>0.9999
PpR24b3 vs. PpR24b5	3.667	No	ns	>0.9999
PpR24b3 vs. PpR24b6	-0.6667	No	ns	>0.9999
PpR24b3 vs. PpR24b7	-5.333	No	ns	>0.9999
PpR24b3 vs. PpR24b8	-7	No	ns	>0.9999
PpR24b3 vs. PpR24b9	15.67	No	ns	>0.9999
PpR24b3 vs. PpR24b10	-18.5	No	ns	>0.9999
PpR24b4 vs. PpR24b5	-4.333	No	ns	>0.9999
PpR24b4 vs. PpR24b6	-8.667	No	ns	>0.9999
PpR24b4 vs. PpR24b7	-13.33	No	ns	>0.9999
PpR24b4 vs. PpR24b8	-15	No	ns	>0.9999
PpR24b4 vs. PpR24b9	7.667	No	ns	>0.9999
PpR24b4 vs. PpR24b10	-26.5	No	ns	>0.9999

PpR24b5 vs. PpR24b6	-4.333	No	ns	>0.9999
PpR24b5 vs. PpR24b7	-9	No	ns	>0.9999
PpR24b5 vs. PpR24b8	-10.67	No	ns	>0.9999
PpR24b5 vs. PpR24b9	12	No	ns	>0.9999
PpR24b5 vs. PpR24b10	-22.17	No	ns	>0.9999
PpR24b6 vs. PpR24b7	-4.667	No	ns	>0.9999
PpR24b6 vs. PpR24b8	-6.333	No	ns	>0.9999
PpR24b6 vs. PpR24b9	16.33	No	ns	>0.9999
PpR24b6 vs. PpR24b10	-17.83	No	ns	>0.9999
PpR24b7 vs. PpR24b8	-1.667	No	ns	>0.9999
PpR24b7 vs. PpR24b9	21	No	ns	>0.9999
PpR24b7 vs. PpR24b10	-13.17	No	ns	>0.9999
PpR24b8 vs. PpR24b9	22.67	No	ns	>0.9999
PpR24b8 vs. PpR24b10	-11.5	No	ns	>0.9999
PpR24b9 vs. PpR24b10	-34.17	No	ns	>0.9999

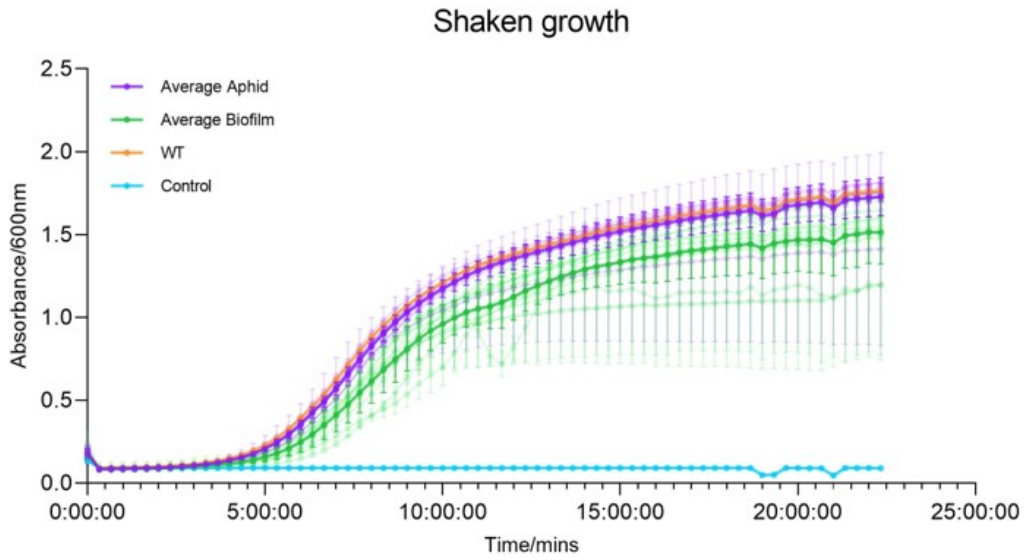

Figure B.2: Evolved isolate and wild-type PpR24 growth in shaken conditions (n=4)

Table B.5: Complete pairwise differences for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment for wild-type and evolved isolate growth in shaken conditions. Kruskal-Wallis test p-value = <0.0001.

Dunn's multiple comparisons test	**Mean rank diff.**	**Sign.?**	**Summary**	**Adjusted P-Value**
WT vs. Control	46.5	No	ns	0.6949
WT vs. PpR24a1	2.333	No	ns	>0.9999
WT vs. PpR24a2	2.667	No	ns	>0.9999
WT vs. PpR24a3	10.17	No	ns	>0.9999
WT vs. PpR24a4	12	No	ns	>0.9999
WT vs. PpR24a5	1.667	No	ns	>0.9999
WT vs. PpR24a6	-7	No	ns	>0.9999
WT vs. PpR24a7	-13.67	No	ns	>0.9999
WT vs. PpR24a8	-14.83	No	ns	>0.9999
WT vs. PpR24a9	2	No	ns	>0.9999
WT vs. PpR24a10	17.17	No	ns	>0.9999
WT vs. PpR24b1	21.5	No	ns	>0.9999
WT vs. PpR24b2	34.5	No	ns	>0.9999
WT vs. PpR24b3	30.83	No	ns	>0.9999

WT vs. PpR24b4	38.5	No	ns	>0.9999
WT vs. PpR24b5	36.5	No	ns	>0.9999
WT vs. PpR24b6	31.83	No	ns	>0.9999
WT vs. PpR24b7	13.33	No	ns	>0.9999
WT vs. PpR24b8	26.5	No	ns	>0.9999
WT vs. PpR24b9	36.17	No	ns	>0.9999
WT vs. PpR24b10	1.333	No	ns	>0.9999
Control vs. PpR24a1	-44.17	No	ns	>0.9999
Control vs. PpR24a2	-43.83	No	ns	>0.9999
Control vs. PpR24a3	-36.33	No	ns	>0.9999
Control vs. PpR24a4	-34.5	No	ns	>0.9999
Control vs. PpR24a5	-44.83	No	ns	0.9769
Control vs. PpR24a6	-53.5	No	ns	0.1481
Control vs. PpR24a7	-60.17	Yes	*	0.0285
Control vs. PpR24a8	-61.33	Yes	*	0.021
Control vs. PpR24a9	-44.5	No	ns	>0.9999
Control vs. PpR24a10	-29.33	No	ns	>0.9999
Control vs. PpR24b1	-25	No	ns	>0.9999
Control vs. PpR24b2	-12	No	ns	>0.9999
Control vs. PpR24b3	-15.67	No	ns	>0.9999
Control vs. PpR24b4	-8	No	ns	>0.9999
Control vs. PpR24b5	-10	No	ns	>0.9999
Control vs. PpR24b6	-14.67	No	ns	>0.9999
Control vs. PpR24b7	-33.17	No	ns	>0.9999
Control vs. PpR24b8	-20	No	ns	>0.9999
Control vs. PpR24b9	-10.33	No	ns	>0.9999
Control vs. PpR24b10	-45.17	No	ns	0.9133
PpR24a1 vs. PpR24a2	0.3333	No	ns	>0.9999
PpR24a1 vs. PpR24a3	7.833	No	ns	>0.9999
PpR24a1 vs. PpR24a4	9.667	No	ns	>0.9999
PpR24a1 vs. PpR24a5	-0.6667	No	ns	>0.9999
PpR24a1 vs. PpR24a6	-9.333	No	ns	>0.9999
PpR24a1 vs. PpR24a7	-16	No	ns	>0.9999
PpR24a1 vs. PpR24a8	-17.17	No	ns	>0.9999

PpR24a1 vs. PpR24a9	-0.3333	No	ns	>0.9999
PpR24a1 vs. PpR24a10	14.83	No	ns	>0.9999
PpR24a1 vs. PpR24b1	19.17	No	ns	>0.9999
PpR24a1 vs. PpR24b2	32.17	No	ns	>0.9999
PpR24a1 vs. PpR24b3	28.5	No	ns	>0.9999
PpR24a1 vs. PpR24b4	36.17	No	ns	>0.9999
PpR24a1 vs. PpR24b5	34.17	No	ns	>0.9999
PpR24a1 vs. PpR24b6	29.5	No	ns	>0.9999
PpR24a1 vs. PpR24b7	11	No	ns	>0.9999
PpR24a1 vs. PpR24b8	24.17	No	ns	>0.9999
PpR24a1 vs. PpR24b9	33.83	No	ns	>0.9999
PpR24a1 vs. PpR24b10	-1	No	ns	>0.9999
PpR24a2 vs. PpR24a3	7.5	No	ns	>0.9999
PpR24a2 vs. PpR24a4	9.333	No	ns	>0.9999
PpR24a2 vs. PpR24a5	-1	No	ns	>0.9999
PpR24a2 vs. PpR24a6	-9.667	No	ns	>0.9999
PpR24a2 vs. PpR24a7	-16.33	No	ns	>0.9999
PpR24a2 vs. PpR24a8	-17.5	No	ns	>0.9999
PpR24a2 vs. PpR24a9	-0.6667	No	ns	>0.9999
PpR24a2 vs. PpR24a10	14.5	No	ns	>0.9999
PpR24a2 vs. PpR24b1	18.83	No	ns	>0.9999
PpR24a2 vs. PpR24b2	31.83	No	ns	>0.9999
PpR24a2 vs. PpR24b3	28.17	No	ns	>0.9999
PpR24a2 vs. PpR24b4	35.83	No	ns	>0.9999
PpR24a2 vs. PpR24b5	33.83	No	ns	>0.9999
PpR24a2 vs. PpR24b6	29.17	No	ns	>0.9999
PpR24a2 vs. PpR24b7	10.67	No	ns	>0.9999
PpR24a2 vs. PpR24b8	23.83	No	ns	>0.9999
PpR24a2 vs. PpR24b9	33.5	No	ns	>0.9999
PpR24a2 vs. PpR24b10	-1.333	No	ns	>0.9999
PpR24a3 vs. PpR24a4	1.833	No	ns	>0.9999
PpR24a3 vs. PpR24a5	-8.5	No	ns	>0.9999
PpR24a3 vs. PpR24a6	-17.17	No	ns	>0.9999
PpR24a3 vs. PpR24a7	-23.83	No	ns	>0.9999

PpR24a3 vs. PpR24a8	-25	No	ns	>0.9999
PpR24a3 vs. PpR24a9	-8.167	No	ns	>0.9999
PpR24a3 vs. PpR24a10	7	No	ns	>0.9999
PpR24a3 vs. PpR24b1	11.33	No	ns	>0.9999
PpR24a3 vs. PpR24b2	24.33	No	ns	>0.9999
PpR24a3 vs. PpR24b3	20.67	No	ns	>0.9999
PpR24a3 vs. PpR24b4	28.33	No	ns	>0.9999
PpR24a3 vs. PpR24b5	26.33	No	ns	>0.9999
PpR24a3 vs. PpR24b6	21.67	No	ns	>0.9999
PpR24a3 vs. PpR24b7	3.167	No	ns	>0.9999
PpR24a3 vs. PpR24b8	16.33	No	ns	>0.9999
PpR24a3 vs. PpR24b9	26	No	ns	>0.9999
PpR24a3 vs. PpR24b10	-8.833	No	ns	>0.9999
PpR24a4 vs. PpR24a5	-10.33	No	ns	>0.9999
PpR24a4 vs. PpR24a6	-19	No	ns	>0.9999
PpR24a4 vs. PpR24a7	-25.67	No	ns	>0.9999
PpR24a4 vs. PpR24a8	-26.83	No	ns	>0.9999
PpR24a4 vs. PpR24a9	-10	No	ns	>0.9999
PpR24a4 vs. PpR24a10	5.167	No	ns	>0.9999
PpR24a4 vs. PpR24b1	9.5	No	ns	>0.9999
PpR24a4 vs. PpR24b2	22.5	No	ns	>0.9999
PpR24a4 vs. PpR24b3	18.83	No	ns	>0.9999
PpR24a4 vs. PpR24b4	26.5	No	ns	>0.9999
PpR24a4 vs. PpR24b5	24.5	No	ns	>0.9999
PpR24a4 vs. PpR24b6	19.83	No	ns	>0.9999
PpR24a4 vs. PpR24b7	1.333	No	ns	>0.9999
PpR24a4 vs. PpR24b8	14.5	No	ns	>0.9999
PpR24a4 vs. PpR24b9	24.17	No	ns	>0.9999
PpR24a4 vs. PpR24b10	-10.67	No	ns	>0.9999
PpR24a5 vs. PpR24a6	-8.667	No	ns	>0.9999
PpR24a5 vs. PpR24a7	-15.33	No	ns	>0.9999
PpR24a5 vs. PpR24a8	-16.5	No	ns	>0.9999
PpR24a5 vs. PpR24a9	0.3333	No	ns	>0.9999
PpR24a5 vs. PpR24a10	15.5	No	ns	>0.9999

PpR24a5 vs. PpR24b1	19.83	No	ns	>0.9999
PpR24a5 vs. PpR24b2	32.83	No	ns	>0.9999
PpR24a5 vs. PpR24b3	29.17	No	ns	>0.9999
PpR24a5 vs. PpR24b4	36.83	No	ns	>0.9999
PpR24a5 vs. PpR24b5	34.83	No	ns	>0.9999
PpR24a5 vs. PpR24b6	30.17	No	ns	>0.9999
PpR24a5 vs. PpR24b7	11.67	No	ns	>0.9999
PpR24a5 vs. PpR24b8	24.83	No	ns	>0.9999
PpR24a5 vs. PpR24b9	34.5	No	ns	>0.9999
PpR24a5 vs. PpR24b10	-0.3333	No	ns	>0.9999
PpR24a6 vs. PpR24a7	-6.667	No	ns	>0.9999
PpR24a6 vs. PpR24a8	-7.833	No	ns	>0.9999
PpR24a6 vs. PpR24a9	9	No	ns	>0.9999
PpR24a6 vs. PpR24a10	24.17	No	ns	>0.9999
PpR24a6 vs. PpR24b1	28.5	No	ns	>0.9999
PpR24a6 vs. PpR24b2	41.5	No	ns	>0.9999
PpR24a6 vs. PpR24b3	37.83	No	ns	>0.9999
PpR24a6 vs. PpR24b4	45.5	No	ns	0.8535
PpR24a6 vs. PpR24b5	43.5	No	ns	>0.9999
PpR24a6 vs. PpR24b6	38.83	No	ns	>0.9999
PpR24a6 vs. PpR24b7	20.33	No	ns	>0.9999
PpR24a6 vs. PpR24b8	33.5	No	ns	>0.9999
PpR24a6 vs. PpR24b9	43.17	No	ns	>0.9999
PpR24a6 vs. PpR24b10	8.333	No	ns	>0.9999
PpR24a7 vs. PpR24a8	-1.167	No	ns	>0.9999
PpR24a7 vs. PpR24a9	15.67	No	ns	>0.9999
PpR24a7 vs. PpR24a10	30.83	No	ns	>0.9999
PpR24a7 vs. PpR24b1	35.17	No	ns	>0.9999
PpR24a7 vs. PpR24b2	48.17	No	ns	0.4891
PpR24a7 vs. PpR24b3	44.5	No	ns	>0.9999
PpR24a7 vs. PpR24b4	52.17	No	ns	0.2017
PpR24a7 vs. PpR24b5	50.17	No	ns	0.3165
PpR24a7 vs. PpR24b6	45.5	No	ns	0.8535
PpR24a7 vs. PpR24b7	27	No	ns	>0.9999

PpR24a7 vs. PpR24b8	40.17	No	ns	>0.9999
PpR24a7 vs. PpR24b9	49.83	No	ns	0.3407
PpR24a7 vs. PpR24b10	15	No	ns	>0.9999
PpR24a8 vs. PpR24a9	16.83	No	ns	>0.9999
PpR24a8 vs. PpR24a10	32	No	ns	>0.9999
PpR24a8 vs. PpR24b1	36.33	No	ns	>0.9999
PpR24a8 vs. PpR24b2	49.33	No	ns	0.3802
PpR24a8 vs. PpR24b3	45.67	No	ns	0.825
PpR24a8 vs. PpR24b4	53.33	No	ns	0.154
PpR24a8 vs. PpR24b5	51.33	No	ns	0.2438
PpR24a8 vs. PpR24b6	46.67	No	ns	0.6712
PpR24a8 vs. PpR24b7	28.17	No	ns	>0.9999
PpR24a8 vs. PpR24b8	41.33	No	ns	>0.9999
PpR24a8 vs. PpR24b9	51	No	ns	0.2628
PpR24a8 vs. PpR24b10	16.17	No	ns	>0.9999
PpR24a9 vs. PpR24a10	15.17	No	ns	>0.9999
PpR24a9 vs. PpR24b1	19.5	No	ns	>0.9999
PpR24a9 vs. PpR24b2	32.5	No	ns	>0.9999
PpR24a9 vs. PpR24b3	28.83	No	ns	>0.9999
PpR24a9 vs. PpR24b4	36.5	No	ns	>0.9999
PpR24a9 vs. PpR24b5	34.5	No	ns	>0.9999
PpR24a9 vs. PpR24b6	29.83	No	ns	>0.9999
PpR24a9 vs. PpR24b7	11.33	No	ns	>0.9999
PpR24a9 vs. PpR24b8	24.5	No	ns	>0.9999
PpR24a9 vs. PpR24b9	34.17	No	ns	>0.9999
PpR24a9 vs. PpR24b10	-0.6667	No	ns	>0.9999
PpR24a10 vs. PpR24b1	4.333	No	ns	>0.9999
PpR24a10 vs. PpR24b2	17.33	No	ns	>0.9999
PpR24a10 vs. PpR24b3	13.67	No	ns	>0.9999
PpR24a10 vs. PpR24b4	21.33	No	ns	>0.9999
PpR24a10 vs. PpR24b5	19.33	No	ns	>0.9999
PpR24a10 vs. PpR24b6	14.67	No	ns	>0.9999
PpR24a10 vs. PpR24b7	-3.833	No	ns	>0.9999
PpR24a10 vs. PpR24b8	9.333	No	ns	>0.9999

PpR24a10 vs. PpR24b9	19	No	ns	>0.9999
PpR24a10 vs. PpR24b10	-15.83	No	ns	>0.9999
PpR24b1 vs. PpR24b2	13	No	ns	>0.9999
PpR24b1 vs. PpR24b3	9.333	No	ns	>0.9999
PpR24b1 vs. PpR24b4	17	No	ns	>0.9999
PpR24b1 vs. PpR24b5	15	No	ns	>0.9999
PpR24b1 vs. PpR24b6	10.33	No	ns	>0.9999
PpR24b1 vs. PpR24b7	-8.167	No	ns	>0.9999
PpR24b1 vs. PpR24b8	5	No	ns	>0.9999
PpR24b1 vs. PpR24b9	14.67	No	ns	>0.9999
PpR24b1 vs. PpR24b10	-20.17	No	ns	>0.9999
PpR24b2 vs. PpR24b3	-3.667	No	ns	>0.9999
PpR24b2 vs. PpR24b4	4	No	ns	>0.9999
PpR24b2 vs. PpR24b5	2	No	ns	>0.9999
PpR24b2 vs. PpR24b6	-2.667	No	ns	>0.9999
PpR24b2 vs. PpR24b7	-21.17	No	ns	>0.9999
PpR24b2 vs. PpR24b8	-8	No	ns	>0.9999
PpR24b2 vs. PpR24b9	1.667	No	ns	>0.9999
PpR24b2 vs. PpR24b10	-33.17	No	ns	>0.9999
PpR24b3 vs. PpR24b4	7.667	No	ns	>0.9999
PpR24b3 vs. PpR24b5	5.667	No	ns	>0.9999
PpR24b3 vs. PpR24b6	1	No	ns	>0.9999
PpR24b3 vs. PpR24b7	-17.5	No	ns	>0.9999
PpR24b3 vs. PpR24b8	-4.333	No	ns	>0.9999
PpR24b3 vs. PpR24b9	5.333	No	ns	>0.9999
PpR24b3 vs. PpR24b10	-29.5	No	ns	>0.9999
PpR24b4 vs. PpR24b5	-2	No	ns	>0.9999
PpR24b4 vs. PpR24b6	-6.667	No	ns	>0.9999
PpR24b4 vs. PpR24b7	-25.17	No	ns	>0.9999
PpR24b4 vs. PpR24b8	-12	No	ns	>0.9999
PpR24b4 vs. PpR24b9	-2.333	No	ns	>0.9999
PpR24b4 vs. PpR24b10	-37.17	No	ns	>0.9999
PpR24b5 vs. PpR24b6	-4.667	No	ns	>0.9999
PpR24b5 vs. PpR24b7	-23.17	No	ns	>0.9999

PpR24b5 vs. PpR24b8	-10	No	ns	>0.9999
PpR24b5 vs. PpR24b9	-0.3333	No	ns	>0.9999
PpR24b5 vs. PpR24b10	-35.17	No	ns	>0.9999
PpR24b6 vs. PpR24b7	-18.5	No	ns	>0.9999
PpR24b6 vs. PpR24b8	-5.333	No	ns	>0.9999
PpR24b6 vs. PpR24b9	4.333	No	ns	>0.9999
PpR24b6 vs. PpR24b10	-30.5	No	ns	>0.9999
PpR24b7 vs. PpR24b8	13.17	No	ns	>0.9999
PpR24b7 vs. PpR24b9	22.83	No	ns	>0.9999
PpR24b7 vs. PpR24b10	-12	No	ns	>0.9999
PpR24b8 vs. PpR24b9	9.667	No	ns	>0.9999
PpR24b8 vs. PpR24b10	-25.17	No	ns	>0.9999
PpR24b9 vs. PpR24b10	-34.83	No	ns	>0.9999

B.1.4 Motility trade-offs

Table B.6: Complete pairwise differences for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment for wild-type and evolved isolate swimming motility. Kruskal-Wallis test p-value = <0.0001.

Dunn's multiple comparisons test	Mean rank diff.	Sign.?	Summary	Adjusted P-Value
Control vs. WT	-143	Yes	****	<0.0001
Control vs. PpR24a1	-124.9	Yes	***	0.0005
Control vs. PpR24a2	-132.8	Yes	***	0.0001
Control vs. PpR24a3	-125.8	Yes	***	0.0004
Control vs. PpR24a4	-138.8	Yes	****	<0.0001
Control vs. PpR24a5	-119.2	Yes	**	0.0014
Control vs. PpR24a6	-128	Yes	**	0.0032
Control vs. PpR24a7	-129.3	Yes	***	0.0002
Control vs. PpR24a8	-133.9	Yes	****	<0.0001
Control vs. PpR24a9	-135.5	Yes	****	<0.0001
Control vs. PpR24a10	-140.6	Yes	***	0.0001
Control vs. PpR24b1	-94.33	No	ns	0.0786
Control vs. PpR24b2	-21	No	ns	>0.9999
Control vs. PpR24b3	-74.56	No	ns	>0.9999
Control vs. PpR24b4	-51.67	No	ns	>0.9999
Control vs. PpR24b5	-107	Yes	*	0.0112
Control vs. PpR24b6	-19.89	No	ns	>0.9999
Control vs. PpR24b7	-64	No	ns	>0.9999
Control vs. PpR24b8	-42.78	No	ns	>0.9999
Control vs. PpR24b9	-78.67	No	ns	0.6494
Control vs. PpR24b10	-41.11	No	ns	>0.9999
WT vs. PpR24a1	18.06	No	ns	>0.9999
WT vs. PpR24a2	10.22	No	ns	>0.9999
WT vs. PpR24a3	17.22	No	ns	>0.9999
WT vs. PpR24a4	4.222	No	ns	>0.9999
WT vs. PpR24a5	23.78	No	ns	>0.9999
WT vs. PpR24a6	15	No	ns	>0.9999
WT vs. PpR24a7	13.67	No	ns	>0.9999

WT vs. PpR24a8	9.111	No	ns	>0.9999
WT vs. PpR24a9	7.5	No	ns	>0.9999
WT vs. PpR24a10	2.429	No	ns	>0.9999
WT vs. PpR24b1	48.67	No	ns	>0.9999
WT vs. PpR24b2	122	Yes	***	0.0008
WT vs. PpR24b3	68.44	No	ns	>0.9999
WT vs. PpR24b4	91.33	No	ns	0.1208
WT vs. PpR24b5	36	No	ns	>0.9999
WT vs. PpR24b6	123.1	Yes	***	0.0007
WT vs. PpR24b7	79	No	ns	0.623
WT vs. PpR24b8	100.2	Yes	*	0.0326
WT vs. PpR24b9	64.33	No	ns	>0.9999
WT vs. PpR24b10	101.9	Yes	*	0.0252
PpR24a1 vs. PpR24a2	-7.833	No	ns	>0.9999
PpR24a1 vs. PpR24a3	-0.8333	No	ns	>0.9999
PpR24a1 vs. PpR24a4	-13.83	No	ns	>0.9999
PpR24a1 vs. PpR24a5	5.722	No	ns	>0.9999
PpR24a1 vs. PpR24a6	-3.056	No	ns	>0.9999
PpR24a1 vs. PpR24a7	-4.389	No	ns	>0.9999
PpR24a1 vs. PpR24a8	-8.944	No	ns	>0.9999
PpR24a1 vs. PpR24a9	-10.56	No	ns	>0.9999
PpR24a1 vs. PpR24a10	-15.63	No	ns	>0.9999
PpR24a1 vs. PpR24b1	30.61	No	ns	>0.9999
PpR24a1 vs. PpR24b2	103.9	Yes	*	0.0182
PpR24a1 vs. PpR24b3	50.39	No	ns	>0.9999
PpR24a1 vs. PpR24b4	73.28	No	ns	>0.9999
PpR24a1 vs. PpR24b5	17.94	No	ns	>0.9999
PpR24a1 vs. PpR24b6	105.1	Yes	*	0.0153
PpR24a1 vs. PpR24b7	60.94	No	ns	>0.9999
PpR24a1 vs. PpR24b8	82.17	No	ns	0.4169
PpR24a1 vs. PpR24b9	46.28	No	ns	>0.9999
PpR24a1 vs. PpR24b10	83.83	No	ns	0.3357
PpR24a2 vs. PpR24a3	7	No	ns	>0.9999
PpR24a2 vs. PpR24a4	-6	No	ns	>0.9999

PpR24a2 vs. PpR24a5	13.56	No	ns	>0.9999
PpR24a2 vs. PpR24a6	4.778	No	ns	>0.9999
PpR24a2 vs. PpR24a7	3.444	No	ns	>0.9999
PpR24a2 vs. PpR24a8	-1.111	No	ns	>0.9999
PpR24a2 vs. PpR24a9	-2.722	No	ns	>0.9999
PpR24a2 vs. PpR24a10	-7.794	No	ns	>0.9999
PpR24a2 vs. PpR24b1	38.44	No	ns	>0.9999
PpR24a2 vs. PpR24b2	111.8	Yes	**	0.005
PpR24a2 vs. PpR24b3	58.22	No	ns	>0.9999
PpR24a2 vs. PpR24b4	81.11	No	ns	0.4774
PpR24a2 vs. PpR24b5	25.78	No	ns	>0.9999
PpR24a2 vs. PpR24b6	112.9	Yes	**	0.0042
PpR24a2 vs. PpR24b7	68.78	No	ns	>0.9999
PpR24a2 vs. PpR24b8	90	No	ns	0.1457
PpR24a2 vs. PpR24b9	54.11	No	ns	>0.9999
PpR24a2 vs. PpR24b10	91.67	No	ns	0.1152
PpR24a3 vs. PpR24a4	-13	No	ns	>0.9999
PpR24a3 vs. PpR24a5	6.556	No	ns	>0.9999
PpR24a3 vs. PpR24a6	-2.222	No	ns	>0.9999
PpR24a3 vs. PpR24a7	-3.556	No	ns	>0.9999
PpR24a3 vs. PpR24a8	-8.111	No	ns	>0.9999
PpR24a3 vs. PpR24a9	-9.722	No	ns	>0.9999
PpR24a3 vs. PpR24a10	-14.79	No	ns	>0.9999
PpR24a3 vs. PpR24b1	31.44	No	ns	>0.9999
PpR24a3 vs. PpR24b2	104.8	Yes	*	0.016
PpR24a3 vs. PpR24b3	51.22	No	ns	>0.9999
PpR24a3 vs. PpR24b4	74.11	No	ns	>0.9999
PpR24a3 vs. PpR24b5	18.78	No	ns	>0.9999
PpR24a3 vs. PpR24b6	105.9	Yes	*	0.0134
PpR24a3 vs. PpR24b7	61.78	No	ns	>0.9999
PpR24a3 vs. PpR24b8	83	No	ns	0.3743
PpR24a3 vs. PpR24b9	47.11	No	ns	>0.9999
PpR24a3 vs. PpR24b10	84.67	No	ns	0.3008
PpR24a4 vs. PpR24a5	19.56	No	ns	>0.9999

PpR24a4 vs. PpR24a6	10.78	No	ns	>0.9999
PpR24a4 vs. PpR24a7	9.444	No	ns	>0.9999
PpR24a4 vs. PpR24a8	4.889	No	ns	>0.9999
PpR24a4 vs. PpR24a9	3.278	No	ns	>0.9999
PpR24a4 vs. PpR24a10	-1.794	No	ns	>0.9999
PpR24a4 vs. PpR24b1	44.44	No	ns	>0.9999
PpR24a4 vs. PpR24b2	117.8	Yes	**	0.0018
PpR24a4 vs. PpR24b3	64.22	No	ns	>0.9999
PpR24a4 vs. PpR24b4	87.11	No	ns	0.2168
PpR24a4 vs. PpR24b5	31.78	No	ns	>0.9999
PpR24a4 vs. PpR24b6	118.9	Yes	**	0.0015
PpR24a4 vs. PpR24b7	74.78	No	ns	>0.9999
PpR24a4 vs. PpR24b8	96	No	ns	0.0615
PpR24a4 vs. PpR24b9	60.11	No	ns	>0.9999
PpR24a4 vs. PpR24b10	97.67	Yes	*	0.048
PpR24a5 vs. PpR24a6	-8.778	No	ns	>0.9999
PpR24a5 vs. PpR24a7	-10.11	No	ns	>0.9999
PpR24a5 vs. PpR24a8	-14.67	No	ns	>0.9999
PpR24a5 vs. PpR24a9	-16.28	No	ns	>0.9999
PpR24a5 vs. PpR24a10	-21.35	No	ns	>0.9999
PpR24a5 vs. PpR24b1	24.89	No	ns	>0.9999
PpR24a5 vs. PpR24b2	98.22	Yes	*	0.0442
PpR24a5 vs. PpR24b3	44.67	No	ns	>0.9999
PpR24a5 vs. PpR24b4	67.56	No	ns	>0.9999
PpR24a5 vs. PpR24b5	12.22	No	ns	>0.9999
PpR24a5 vs. PpR24b6	99.33	Yes	*	0.0373
PpR24a5 vs. PpR24b7	55.22	No	ns	>0.9999
PpR24a5 vs. PpR24b8	76.44	No	ns	0.8531
PpR24a5 vs. PpR24b9	40.56	No	ns	>0.9999
PpR24a5 vs. PpR24b10	78.11	No	ns	0.6957
PpR24a6 vs. PpR24a7	-1.333	No	ns	>0.9999
PpR24a6 vs. PpR24a8	-5.889	No	ns	>0.9999
PpR24a6 vs. PpR24a9	-7.5	No	ns	>0.9999
PpR24a6 vs. PpR24a10	-12.57	No	ns	>0.9999

PpR24a6 vs. PpR24b1	33.67	No	ns	>0.9999
PpR24a6 vs. PpR24b2	107	No	ns	0.0643
PpR24a6 vs. PpR24b3	53.44	No	ns	>0.9999
PpR24a6 vs. PpR24b4	76.33	No	ns	>0.9999
PpR24a6 vs. PpR24b5	21	No	ns	>0.9999
PpR24a6 vs. PpR24b6	108.1	No	ns	0.0555
PpR24a6 vs. PpR24b7	64	No	ns	>0.9999
PpR24a6 vs. PpR24b8	85.22	No	ns	0.876
PpR24a6 vs. PpR24b9	49.33	No	ns	>0.9999
PpR24a6 vs. PpR24b10	86.89	No	ns	0.7304
PpR24a7 vs. PpR24a8	-4.556	No	ns	>0.9999
PpR24a7 vs. PpR24a9	-6.167	No	ns	>0.9999
PpR24a7 vs. PpR24a10	-11.24	No	ns	>0.9999
PpR24a7 vs. PpR24b1	35	No	ns	>0.9999
PpR24a7 vs. PpR24b2	108.3	Yes	**	0.009
PpR24a7 vs. PpR24b3	54.78	No	ns	>0.9999
PpR24a7 vs. PpR24b4	77.67	No	ns	0.7348
PpR24a7 vs. PpR24b5	22.33	No	ns	>0.9999
PpR24a7 vs. PpR24b6	109.4	Yes	**	0.0075
PpR24a7 vs. PpR24b7	65.33	No	ns	>0.9999
PpR24a7 vs. PpR24b8	86.56	No	ns	0.2337
PpR24a7 vs. PpR24b9	50.67	No	ns	>0.9999
PpR24a7 vs. PpR24b10	88.22	No	ns	0.1863
PpR24a8 vs. PpR24a9	-1.611	No	ns	>0.9999
PpR24a8 vs. PpR24a10	-6.683	No	ns	>0.9999
PpR24a8 vs. PpR24b1	39.56	No	ns	>0.9999
PpR24a8 vs. PpR24b2	112.9	Yes	**	0.0042
PpR24a8 vs. PpR24b3	59.33	No	ns	>0.9999
PpR24a8 vs. PpR24b4	82.22	No	ns	0.414
PpR24a8 vs. PpR24b5	26.89	No	ns	>0.9999
PpR24a8 vs. PpR24b6	114	Yes	**	0.0035
PpR24a8 vs. PpR24b7	69.89	No	ns	>0.9999
PpR24a8 vs. PpR24b8	91.11	No	ns	0.1246
PpR24a8 vs. PpR24b9	55.22	No	ns	>0.9999

PpR24a8 vs. PpR24b10	92.78	No	ns	0.0983
PpR24a9 vs. PpR24a10	-5.071	No	ns	>0.9999
PpR24a9 vs. PpR24b1	41.17	No	ns	>0.9999
PpR24a9 vs. PpR24b2	114.5	Yes	**	0.0032
PpR24a9 vs. PpR24b3	60.94	No	ns	>0.9999
PpR24a9 vs. PpR24b4	83.83	No	ns	0.3357
PpR24a9 vs. PpR24b5	28.5	No	ns	>0.9999
PpR24a9 vs. PpR24b6	115.6	Yes	**	0.0026
PpR24a9 vs. PpR24b7	71.5	No	ns	>0.9999
PpR24a9 vs. PpR24b8	92.72	No	ns	0.0991
PpR24a9 vs. PpR24b9	56.83	No	ns	>0.9999
PpR24a9 vs. PpR24b10	94.39	No	ns	0.0779
PpR24a10 vs. PpR24b1	46.24	No	ns	>0.9999
PpR24a10 vs. PpR24b2	119.6	Yes	**	0.005
PpR24a10 vs. PpR24b3	66.02	No	ns	>0.9999
PpR24a10 vs. PpR24b4	88.9	No	ns	0.3664
PpR24a10 vs. PpR24b5	33.57	No	ns	>0.9999
PpR24a10 vs. PpR24b6	120.7	Yes	**	0.0042
PpR24a10 vs. PpR24b7	76.57	No	ns	>0.9999
PpR24a10 vs. PpR24b8	97.79	No	ns	0.1184
PpR24a10 vs. PpR24b9	61.9	No	ns	>0.9999
PpR24a10 vs. PpR24b10	99.46	No	ns	0.0948
PpR24b1 vs. PpR24b2	73.33	No	ns	>0.9999
PpR24b1 vs. PpR24b3	19.78	No	ns	>0.9999
PpR24b1 vs. PpR24b4	42.67	No	ns	>0.9999
PpR24b1 vs. PpR24b5	-12.67	No	ns	>0.9999
PpR24b1 vs. PpR24b6	74.44	No	ns	>0.9999
PpR24b1 vs. PpR24b7	30.33	No	ns	>0.9999
PpR24b1 vs. PpR24b8	51.56	No	ns	>0.9999
PpR24b1 vs. PpR24b9	15.67	No	ns	>0.9999
PpR24b1 vs. PpR24b10	53.22	No	ns	>0.9999
PpR24b2 vs. PpR24b3	-53.56	No	ns	>0.9999
PpR24b2 vs. PpR24b4	-30.67	No	ns	>0.9999
PpR24b2 vs. PpR24b5	-86	No	ns	0.2518

PpR24b2 vs. PpR24b6	1.111	No	ns	>0.9999
PpR24b2 vs. PpR24b7	-43	No	ns	>0.9999
PpR24b2 vs. PpR24b8	-21.78	No	ns	>0.9999
PpR24b2 vs. PpR24b9	-57.67	No	ns	>0.9999
PpR24b2 vs. PpR24b10	-20.11	No	ns	>0.9999
PpR24b3 vs. PpR24b4	22.89	No	ns	>0.9999
PpR24b3 vs. PpR24b5	-32.44	No	ns	>0.9999
PpR24b3 vs. PpR24b6	54.67	No	ns	>0.9999
PpR24b3 vs. PpR24b7	10.56	No	ns	>0.9999
PpR24b3 vs. PpR24b8	31.78	No	ns	>0.9999
PpR24b3 vs. PpR24b9	-4.111	No	ns	>0.9999
PpR24b3 vs. PpR24b10	33.44	No	ns	>0.9999
PpR24b4 vs. PpR24b5	-55.33	No	ns	>0.9999
PpR24b4 vs. PpR24b6	31.78	No	ns	>0.9999
PpR24b4 vs. PpR24b7	-12.33	No	ns	>0.9999
PpR24b4 vs. PpR24b8	8.889	No	ns	>0.9999
PpR24b4 vs. PpR24b9	-27	No	ns	>0.9999
PpR24b4 vs. PpR24b10	10.56	No	ns	>0.9999
PpR24b5 vs. PpR24b6	87.11	No	ns	0.2168
PpR24b5 vs. PpR24b7	43	No	ns	>0.9999
PpR24b5 vs. PpR24b8	64.22	No	ns	>0.9999
PpR24b5 vs. PpR24b9	28.33	No	ns	>0.9999
PpR24b5 vs. PpR24b10	65.89	No	ns	>0.9999
PpR24b6 vs. PpR24b7	-44.11	No	ns	>0.9999
PpR24b6 vs. PpR24b8	-22.89	No	ns	>0.9999
PpR24b6 vs. PpR24b9	-58.78	No	ns	>0.9999
PpR24b6 vs. PpR24b10	-21.22	No	ns	>0.9999
PpR24b7 vs. PpR24b8	21.22	No	ns	>0.9999
PpR24b7 vs. PpR24b9	-14.67	No	ns	>0.9999
PpR24b7 vs. PpR24b10	22.89	No	ns	>0.9999
PpR24b8 vs. PpR24b9	-35.89	No	ns	>0.9999
PpR24b8 vs. PpR24b10	1.667	No	ns	>0.9999
PpR24b9 vs. PpR24b10	37.56	No	ns	>0.9999

Table B.7: Complete pairwise differences for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment for wild-type and evolved isolate swarming motility. Kruskal-Wallis test p-value = <0.0001.

Dunn's multiple comparisons test	**Mean rank diff.**	**Sign.?**	**Summary**	**Adjusted P-Value**
Control vs. WT	-101	Yes	*	0.0126
Control vs. PpR24a1	-103.9	Yes	***	0.0008
Control vs. PpR24a2	-120.2	Yes	****	<0.0001
Control vs. PpR24a3	-100	Yes	**	0.0018
Control vs. PpR24a4	-119.3	Yes	****	<0.0001
Control vs. PpR24a5	-109.1	Yes	***	0.0003
Control vs. PpR24a6	-112.4	Yes	***	0.0003
Control vs. PpR24a7	-107.8	Yes	***	0.0007
Control vs. PpR24a8	-111.3	Yes	***	0.0002
Control vs. PpR24a9	-128.3	Yes	****	<0.0001
Control vs. PpR24a10	-105.9	Yes	***	0.0005
Control vs. PpR24b1	-44.5	No	ns	>0.9999
Control vs. PpR24b2	-22.67	No	ns	>0.9999
Control vs. PpR24b3	-41.5	No	ns	>0.9999
Control vs. PpR24b4	-51.5	No	ns	>0.9999
Control vs. PpR24b5	-62.33	No	ns	>0.9999
Control vs. PpR24b6	-11.67	No	ns	>0.9999
Control vs. PpR24b7	-27.5	No	ns	>0.9999
Control vs. PpR24b8	-11.5	No	ns	>0.9999
Control vs. PpR24b9	-48.17	No	ns	>0.9999
Control vs. PpR24b10	-34	No	ns	>0.9999
WT vs. PpR24a1	-2.944	No	ns	>0.9999
WT vs. PpR24a2	-19.22	No	ns	>0.9999
WT vs. PpR24a3	1	No	ns	>0.9999
WT vs. PpR24a4	-18.33	No	ns	>0.9999
WT vs. PpR24a5	-8.056	No	ns	>0.9999
WT vs. PpR24a6	-11.38	No	ns	>0.9999
WT vs. PpR24a7	-6.75	No	ns	>0.9999
WT vs. PpR24a8	-10.33	No	ns	>0.9999
WT vs. PpR24a9	-27.3	No	ns	>0.9999

WT vs. PpR24a10	-4.889	No	ns	>0.9999
WT vs. PpR24b1	56.5	No	ns	>0.9999
WT vs. PpR24b2	78.33	No	ns	0.9866
WT vs. PpR24b3	59.5	No	ns	>0.9999
WT vs. PpR24b4	49.5	No	ns	>0.9999
WT vs. PpR24b5	38.67	No	ns	>0.9999
WT vs. PpR24b6	89.33	No	ns	0.2586
WT vs. PpR24b7	73.5	No	ns	>0.9999
WT vs. PpR24b8	89.5	No	ns	0.2531
WT vs. PpR24b9	52.83	No	ns	>0.9999
WT vs. PpR24b10	67	No	ns	>0.9999
PpR24a1 vs. PpR24a2	-16.28	No	ns	>0.9999
PpR24a1 vs. PpR24a3	3.944	No	ns	>0.9999
PpR24a1 vs. PpR24a4	-15.39	No	ns	>0.9999
PpR24a1 vs. PpR24a5	-5.111	No	ns	>0.9999
PpR24a1 vs. PpR24a6	-8.431	No	ns	>0.9999
PpR24a1 vs. PpR24a7	-3.806	No	ns	>0.9999
PpR24a1 vs. PpR24a8	-7.389	No	ns	>0.9999
PpR24a1 vs. PpR24a9	-24.36	No	ns	>0.9999
PpR24a1 vs. PpR24a10	-1.944	No	ns	>0.9999
PpR24a1 vs. PpR24b1	59.44	No	ns	>0.9999
PpR24a1 vs. PpR24b2	81.28	No	ns	0.2687
PpR24a1 vs. PpR24b3	62.44	No	ns	>0.9999
PpR24a1 vs. PpR24b4	52.44	No	ns	>0.9999
PpR24a1 vs. PpR24b5	41.61	No	ns	>0.9999
PpR24a1 vs. PpR24b6	92.28	No	ns	0.0523
PpR24a1 vs. PpR24b7	76.44	No	ns	0.5205
PpR24a1 vs. PpR24b8	92.44	No	ns	0.051
PpR24a1 vs. PpR24b9	55.78	No	ns	>0.9999
PpR24a1 vs. PpR24b10	69.94	No	ns	>0.9999
PpR24a2 vs. PpR24a3	20.22	No	ns	>0.9999
PpR24a2 vs. PpR24a4	0.8889	No	ns	>0.9999
PpR24a2 vs. PpR24a5	11.17	No	ns	>0.9999
PpR24a2 vs. PpR24a6	7.847	No	ns	>0.9999

PpR24a2 vs. PpR24a7	12.47	No	ns	>0.9999
PpR24a2 vs. PpR24a8	8.889	No	ns	>0.9999
PpR24a2 vs. PpR24a9	-8.078	No	ns	>0.9999
PpR24a2 vs. PpR24a10	14.33	No	ns	>0.9999
PpR24a2 vs. PpR24b1	75.72	No	ns	0.5728
PpR24a2 vs. PpR24b2	97.56	Yes	*	0.0224
PpR24a2 vs. PpR24b3	78.72	No	ns	0.3828
PpR24a2 vs. PpR24b4	68.72	No	ns	>0.9999
PpR24a2 vs. PpR24b5	57.89	No	ns	>0.9999
PpR24a2 vs. PpR24b6	108.6	Yes	**	0.0033
PpR24a2 vs. PpR24b7	92.72	Yes	*	0.0488
PpR24a2 vs. PpR24b8	108.7	Yes	**	0.0032
PpR24a2 vs. PpR24b9	72.06	No	ns	0.9207
PpR24a2 vs. PpR24b10	86.22	No	ns	0.1317
PpR24a3 vs. PpR24a4	-19.33	No	ns	>0.9999
PpR24a3 vs. PpR24a5	-9.056	No	ns	>0.9999
PpR24a3 vs. PpR24a6	-12.38	No	ns	>0.9999
PpR24a3 vs. PpR24a7	-7.75	No	ns	>0.9999
PpR24a3 vs. PpR24a8	-11.33	No	ns	>0.9999
PpR24a3 vs. PpR24a9	-28.3	No	ns	>0.9999
PpR24a3 vs. PpR24a10	-5.889	No	ns	>0.9999
PpR24a3 vs. PpR24b1	55.5	No	ns	>0.9999
PpR24a3 vs. PpR24b2	77.33	No	ns	0.4621
PpR24a3 vs. PpR24b3	58.5	No	ns	>0.9999
PpR24a3 vs. PpR24b4	48.5	No	ns	>0.9999
PpR24a3 vs. PpR24b5	37.67	No	ns	>0.9999
PpR24a3 vs. PpR24b6	88.33	No	ns	0.0961
PpR24a3 vs. PpR24b7	72.5	No	ns	0.8702
PpR24a3 vs. PpR24b8	88.5	No	ns	0.0937
PpR24a3 vs. PpR24b9	51.83	No	ns	>0.9999
PpR24a3 vs. PpR24b10	66	No	ns	>0.9999
PpR24a4 vs. PpR24a5	10.28	No	ns	>0.9999
PpR24a4 vs. PpR24a6	6.958	No	ns	>0.9999
PpR24a4 vs. PpR24a7	11.58	No	ns	>0.9999

PpR24a4 vs. PpR24a8	8	No	ns	>0.9999
PpR24a4 vs. PpR24a9	-8.967	No	ns	>0.9999
PpR24a4 vs. PpR24a10	13.44	No	ns	>0.9999
PpR24a4 vs. PpR24b1	74.83	No	ns	0.6439
PpR24a4 vs. PpR24b2	96.67	Yes	*	0.0259
PpR24a4 vs. PpR24b3	77.83	No	ns	0.4319
PpR24a4 vs. PpR24b4	67.83	No	ns	>0.9999
PpR24a4 vs. PpR24b5	57	No	ns	>0.9999
PpR24a4 vs. PpR24b6	107.7	Yes	**	0.0039
PpR24a4 vs. PpR24b7	91.83	No	ns	0.0561
PpR24a4 vs. PpR24b8	107.8	Yes	**	0.0038
PpR24a4 vs. PpR24b9	71.17	No	ns	>0.9999
PpR24a4 vs. PpR24b10	85.33	No	ns	0.1501
PpR24a5 vs. PpR24a6	-3.319	No	ns	>0.9999
PpR24a5 vs. PpR24a7	1.306	No	ns	>0.9999
PpR24a5 vs. PpR24a8	-2.278	No	ns	>0.9999
PpR24a5 vs. PpR24a9	-19.24	No	ns	>0.9999
PpR24a5 vs. PpR24a10	3.167	No	ns	>0.9999
PpR24a5 vs. PpR24b1	64.56	No	ns	>0.9999
PpR24a5 vs. PpR24b2	86.39	No	ns	0.1285
PpR24a5 vs. PpR24b3	67.56	No	ns	>0.9999
PpR24a5 vs. PpR24b4	57.56	No	ns	>0.9999
PpR24a5 vs. PpR24b5	46.72	No	ns	>0.9999
PpR24a5 vs. PpR24b6	97.39	Yes	*	0.023
PpR24a5 vs. PpR24b7	81.56	No	ns	0.2584
PpR24a5 vs. PpR24b8	97.56	Yes	*	0.0224
PpR24a5 vs. PpR24b9	60.89	No	ns	>0.9999
PpR24a5 vs. PpR24b10	75.06	No	ns	0.6254
PpR24a6 vs. PpR24a7	4.625	No	ns	>0.9999
PpR24a6 vs. PpR24a8	1.042	No	ns	>0.9999
PpR24a6 vs. PpR24a9	-15.93	No	ns	>0.9999
PpR24a6 vs. PpR24a10	6.486	No	ns	>0.9999
PpR24a6 vs. PpR24b1	67.88	No	ns	>0.9999
PpR24a6 vs. PpR24b2	89.71	No	ns	0.1082

PpR24a6 vs. PpR24b3	70.88	No	ns	>0.9999
PpR24a6 vs. PpR24b4	60.88	No	ns	>0.9999
PpR24a6 vs. PpR24b5	50.04	No	ns	>0.9999
PpR24a6 vs. PpR24b6	100.7	Yes	*	0.0198
PpR24a6 vs. PpR24b7	84.88	No	ns	0.2157
PpR24a6 vs. PpR24b8	100.9	Yes	*	0.0193
PpR24a6 vs. PpR24b9	64.21	No	ns	>0.9999
PpR24a6 vs. PpR24b10	78.38	No	ns	0.5176
PpR24a7 vs. PpR24a8	-3.583	No	ns	>0.9999
PpR24a7 vs. PpR24a9	-20.55	No	ns	>0.9999
PpR24a7 vs. PpR24a10	1.861	No	ns	>0.9999
PpR24a7 vs. PpR24b1	63.25	No	ns	>0.9999
PpR24a7 vs. PpR24b2	85.08	No	ns	0.2095
PpR24a7 vs. PpR24b3	66.25	No	ns	>0.9999
PpR24a7 vs. PpR24b4	56.25	No	ns	>0.9999
PpR24a7 vs. PpR24b5	45.42	No	ns	>0.9999
PpR24a7 vs. PpR24b6	96.08	Yes	*	0.0414
PpR24a7 vs. PpR24b7	80.25	No	ns	0.4046
PpR24a7 vs. PpR24b8	96.25	Yes	*	0.0403
PpR24a7 vs. PpR24b9	59.58	No	ns	>0.9999
PpR24a7 vs. PpR24b10	73.75	No	ns	0.9305
PpR24a8 vs. PpR24a9	-16.97	No	ns	>0.9999
PpR24a8 vs. PpR24a10	5.444	No	ns	>0.9999
PpR24a8 vs. PpR24b1	66.83	No	ns	>0.9999
PpR24a8 vs. PpR24b2	88.67	No	ns	0.0914
PpR24a8 vs. PpR24b3	69.83	No	ns	>0.9999
PpR24a8 vs. PpR24b4	59.83	No	ns	>0.9999
PpR24a8 vs. PpR24b5	49	No	ns	>0.9999
PpR24a8 vs. PpR24b6	99.67	Yes	*	0.0157
PpR24a8 vs. PpR24b7	83.83	No	ns	0.1867
PpR24a8 vs. PpR24b8	99.83	Yes	*	0.0153
PpR24a8 vs. PpR24b9	63.17	No	ns	>0.9999
PpR24a8 vs. PpR24b10	77.33	No	ns	0.4621
PpR24a9 vs. PpR24a10	22.41	No	ns	>0.9999

PpR24a9 vs. PpR24b1	83.8	No	ns	0.1459
PpR24a9 vs. PpR24b2	105.6	Yes	**	0.0038
PpR24a9 vs. PpR24b3	86.8	No	ns	0.0924
PpR24a9 vs. PpR24b4	76.8	No	ns	0.4009
PpR24a9 vs. PpR24b5	65.97	No	ns	>0.9999
PpR24a9 vs. PpR24b6	116.6	Yes	***	0.0005
PpR24a9 vs. PpR24b7	100.8	Yes	**	0.0091
PpR24a9 vs. PpR24b8	116.8	Yes	***	0.0004
PpR24a9 vs. PpR24b9	80.13	No	ns	0.2501
PpR24a9 vs. PpR24b10	94.3	Yes	*	0.0278
PpR24a10 vs. PpR24b1	61.39	No	ns	>0.9999
PpR24a10 vs. PpR24b2	83.22	No	ns	0.2039
PpR24a10 vs. PpR24b3	64.39	No	ns	>0.9999
PpR24a10 vs. PpR24b4	54.39	No	ns	>0.9999
PpR24a10 vs. PpR24b5	43.56	No	ns	>0.9999
PpR24a10 vs. PpR24b6	94.22	Yes	*	0.0385
PpR24a10 vs. PpR24b7	78.39	No	ns	0.4006
PpR24a10 vs. PpR24b8	94.39	Yes	*	0.0375
PpR24a10 vs. PpR24b9	57.72	No	ns	>0.9999
PpR24a10 vs. PpR24b10	71.89	No	ns	0.9404
PpR24b1 vs. PpR24b2	21.83	No	ns	>0.9999
PpR24b1 vs. PpR24b3	3	No	ns	>0.9999
PpR24b1 vs. PpR24b4	-7	No	ns	>0.9999
PpR24b1 vs. PpR24b5	-17.83	No	ns	>0.9999
PpR24b1 vs. PpR24b6	32.83	No	ns	>0.9999
PpR24b1 vs. PpR24b7	17	No	ns	>0.9999
PpR24b1 vs. PpR24b8	33	No	ns	>0.9999
PpR24b1 vs. PpR24b9	-3.667	No	ns	>0.9999
PpR24b1 vs. PpR24b10	10.5	No	ns	>0.9999
PpR24b2 vs. PpR24b3	-18.83	No	ns	>0.9999
PpR24b2 vs. PpR24b4	-28.83	No	ns	>0.9999
PpR24b2 vs. PpR24b5	-39.67	No	ns	>0.9999
PpR24b2 vs. PpR24b6	11	No	ns	>0.9999
PpR24b2 vs. PpR24b7	-4.833	No	ns	>0.9999

PpR24b2 vs. PpR24b8	11.17	No	ns	>0.9999
PpR24b2 vs. PpR24b9	-25.5	No	ns	>0.9999
PpR24b2 vs. PpR24b10	-11.33	No	ns	>0.9999
PpR24b3 vs. PpR24b4	-10	No	ns	>0.9999
PpR24b3 vs. PpR24b5	-20.83	No	ns	>0.9999
PpR24b3 vs. PpR24b6	29.83	No	ns	>0.9999
PpR24b3 vs. PpR24b7	14	No	ns	>0.9999
PpR24b3 vs. PpR24b8	30	No	ns	>0.9999
PpR24b3 vs. PpR24b9	-6.667	No	ns	>0.9999
PpR24b3 vs. PpR24b10	7.5	No	ns	>0.9999
PpR24b4 vs. PpR24b5	-10.83	No	ns	>0.9999
PpR24b4 vs. PpR24b6	39.83	No	ns	>0.9999
PpR24b4 vs. PpR24b7	24	No	ns	>0.9999
PpR24b4 vs. PpR24b8	40	No	ns	>0.9999
PpR24b4 vs. PpR24b9	3.333	No	ns	>0.9999
PpR24b4 vs. PpR24b10	17.5	No	ns	>0.9999
PpR24b5 vs. PpR24b6	50.67	No	ns	>0.9999
PpR24b5 vs. PpR24b7	34.83	No	ns	>0.9999
PpR24b5 vs. PpR24b8	50.83	No	ns	>0.9999
PpR24b5 vs. PpR24b9	14.17	No	ns	>0.9999
PpR24b5 vs. PpR24b10	28.33	No	ns	>0.9999
PpR24b6 vs. PpR24b7	-15.83	No	ns	>0.9999
PpR24b6 vs. PpR24b8	0.1667	No	ns	>0.9999
PpR24b6 vs. PpR24b9	-36.5	No	ns	>0.9999
PpR24b6 vs. PpR24b10	-22.33	No	ns	>0.9999
PpR24b7 vs. PpR24b8	16	No	ns	>0.9999
PpR24b7 vs. PpR24b9	-20.67	No	ns	>0.9999
PpR24b7 vs. PpR24b10	-6.5	No	ns	>0.9999
PpR24b8 vs. PpR24b9	-36.67	No	ns	>0.9999
PpR24b8 vs. PpR24b10	-22.5	No	ns	>0.9999
PpR24b9 vs. PpR24b10	14.17	No	ns	>0.9999

B.1.5 Plant persistence

Table B.8: Complete pairwise differences for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment for wild-type PpR24, PpR24a1, and PpR24b4 plant persistence. Kruskal-Wallis test p-value = 0.0007.

Dunn's multiple comparisons test	**Mean rank diff.**	**Sign.?**	**Summary**	**Adjusted P-Value**
Control vs. WT	-24.65	Yes	**	0.0038
Control vs. PpR24a1	-24.22	Yes	**	0.0047
Control vs. PpR24b4	-8.685	No	ns	>0.9999
WT vs. PpR24a1	0.4259	No	ns	>0.9999
WT vs. PpR24b4	15.96	No	ns	0.1613
PpR24a1 vs. PpR24b4	15.54	No	ns	0.1874

Appendix C

Chapter 5 Additional Data

C.1 SPME GC-MS VOC pairwise comparisons

Table C.1: Complete pairwise differences of isolates PpR24, PpR24a1, PpR24b4, and control for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment for 2-methylpentane. Kruskal-Wallis test p-value = 0.0012.

Dunn's multiple comparisons test	Mean rank diff.	Sign.?	Summary	Adjusted P-Value
Control vs. PpR24	9	Yes	*	0.0451
Control vs. PpR24a1	9.25	Yes	*	0.036
Control vs. PpR24b4	2.75	No	ns	>0.9999
PpR24 vs. PpR24a1	0.25	No	ns	>0.9999
PpR24 vs. PpR24b4	-6.25	No	ns	0.3803
PpR24a1 vs. PpR24b4	-6.5	No	ns	0.3211

Table C.2: Complete pairwise differences of isolates PpR24, PpR24a1, PpR24b4, and control for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment for dimethyl disulphide. Kruskal-Wallis test p-value = 0.0008.

Dunn's multiple comparisons test	Mean rank diff.	Sign.?	Summary	Adjusted P-Value
Control vs. PpR24	-7.75	No	ns	0.1223
Control vs. PpR24a1	-5.25	No	ns	0.697
Control vs. PpR24b4	-11	Yes	**	0.006
PpR24 vs. PpR24a1	2.5	No	ns	>0.9999
PpR24 vs. PpR24b4	-3.25	No	ns	>0.9999
PpR24a1 vs. PpR24b4	-5.75	No	ns	0.5118

Table C.3: Complete pairwise differences of isolates PpR24, PpR24a1, PpR24b4, and control for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment for methanethiol. Kruskal-Wallis test p-value = 0.0002.

Dunn's multiple comparisons test	**Mean rank diff.**	**Sign.?**	**Summary**	**Adjusted P-Value**
Control vs. PpR24	-6.25	No	ns	0.3686
Control vs. PpR24a1	-6	No	ns	0.4354
Control vs. PpR24b4	-11.75	Yes	**	0.0026
PpR24 vs. PpR24a1	0.25	No	ns	>0.9999
PpR24 vs. PpR24b4	-5.5	No	ns	0.5987
PpR24a1 vs. PpR24b4	-5.75	No	ns	0.5118

Table C.4: Complete pairwise differences of isolates PpR24, PpR24a1, PpR24b4, and control for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment for acetone. Kruskal-Wallis test p-value = 0.0199.

Dunn's multiple comparisons test	**Mean rank diff.**	**Sign.?**	**Summary**	**Adjusted P-Value**
Control vs. PpR24	8.25	No	ns	0.0856
Control vs. PpR24a1	7.5	No	ns	0.1554
Control vs. PpR24b4	8.25	No	ns	0.0856
PpR24 vs. PpR24a1	-0.75	No	ns	>0.9999
PpR24 vs. PpR24b4	0	No	ns	>0.9999
PpR24a1 vs. PpR24b4	0.75	No	ns	>0.9999

Table C.5: Complete pairwise differences of isolates PpR24, PpR24a1, PpR24b4, and control for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment for 1-nonene. Kruskal-Wallis test p-value = <0.0001.

Dunn's multiple comparisons test	**Mean rank diff.**	**Sign.?**	**Summary**	**Adjusted P-Value**
Control vs. PpR24	-10.75	Yes	**	0.0078
Control vs. PpR24a1	-9.25	Yes	*	0.0338
Control vs. PpR24b4	-4	No	ns	>0.9999
PpR24 vs. PpR24a1	1.5	No	ns	>0.9999
PpR24 vs. PpR24b4	6.75	No	ns	0.2603
PpR24a1 vs. PpR24b4	5.25	No	ns	0.697

Table C.6: Complete pairwise differences of isolates PpR24, PpR24a1, PpR24b4, and control for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment for dimethyl sulphide. Kruskal-Wallis test p-value = <0.0001.

Dunn's multiple comparisons test	**Mean rank diff.**	**Sign.?**	**Summary**	**Adjusted P-Value**
Control vs. PpR24	-10.25	Yes	*	0.013
Control vs. PpR24a1	-9.75	Yes	*	0.0212
Control vs. PpR24b4	-4	No	ns	>0.9999
PpR24 vs. PpR24a1	0.5	No	ns	>0.9999
PpR24 vs. PpR24b4	6.25	No	ns	0.3686
PpR24a1 vs. PpR24b4	5.75	No	ns	0.5118

Table C.7: Complete pairwise differences of isolates PpR24, PpR24a1, PpR24b4, and control for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment for 1-decene. Kruskal-Wallis test p-value = <0.0001.

Dunn's multiple comparisons test	**Mean rank diff.**	**Sign.?**	**Summary**	**Adjusted P-Value**
Control vs. PpR24	-10.25	Yes	*	0.0136
Control vs. PpR24a1	-9.75	Yes	*	0.0221
Control vs. PpR24b4	-4	No	ns	>0.9999
PpR24 vs. PpR24a1	0.5	No	ns	>0.9999
PpR24 vs. PpR24b4	6.25	No	ns	0.3756
PpR24a1 vs. PpR24b4	5.75	No	ns	0.5202

Table C.8: Complete pairwise differences of isolates PpR24, PpR24a1, PpR24b4, and control for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment for 1-undecene. Kruskal-Wallis test p-value = <0.0001.

Dunn's multiple comparisons test	**Mean rank diff.**	**Sign.?**	**Summary**	**Adjusted P-Value**
Control vs. PpR24	-10.25	Yes	*	0.014
Control vs. PpR24a1	-9.75	Yes	*	0.0227
Control vs. PpR24b4	-4	No	ns	>0.9999
PpR24 vs. PpR24a1	0.5	No	ns	>0.9999
PpR24 vs. PpR24b4	6.25	No	ns	0.3803
PpR24a1 vs. PpR24b4	5.75	No	ns	0.5258

Table C.9: Complete pairwise differences of isolates PpR24, PpR24a1, PpR24b4, and control for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment for undecane. Kruskal-Wallis test p-value = 0.9999.

Dunn's multiple comparisons test	**Mean rank diff.**	**Sign.?**	**Summary**	**Adjusted P-Value**
Contol vs. PpR24	0.25	No	ns	>0.9999
Contol vs. PpR24a1	0.25	No	ns	>0.9999
Contol vs. PpR24b4	0.5	No	ns	>0.9999
PpR24 vs. PpR24a1	0	No	ns	>0.9999
PpR24 vs. PpR24b4	0.25	No	ns	>0.9999
PpR24a1 vs. PpR24b4	0.25	No	ns	>0.9999

Table C.10: Complete pairwise differences of isolates PpR24, PpR24a1, PpR24b4, and control for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment for dodecane. Kruskal-Wallis test p-value = 0.9977.

Dunn's multiple comparisons test	**Mean rank diff.**	**Sign.?**	**Summary**	**Adjusted P-Value**
Contol vs. PpR24	0.5	No	ns	>0.9999
Contol vs. PpR24a1	0.75	No	ns	>0.9999
Contol vs. PpR24b4	0.75	No	ns	>0.9999
PpR24 vs. PpR24a1	0.25	No	ns	>0.9999
PpR24 vs. PpR24b4	0.25	No	ns	>0.9999
PpR24a1 vs. PpR24b4	0	No	ns	>0.9999

Table C.11: Complete pairwise differences of isolates PpR24, PpR24a1, PpR24b4, and control for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment for tridecane. Kruskal-Wallis test p-value = 0.9999.

Dunn's multiple comparisons test	**Mean rank diff.**	**Sign.?**	**Summary**	**Adjusted P-Value**
Control vs. PpR24	0.25	No	ns	>0.9999
Control vs. PpR24a1	0.25	No	ns	>0.9999
Control vs. PpR24b4	0.5	No	ns	>0.9999
PpR24 vs. PpR24a1	0	No	ns	>0.9999
PpR24 vs. PpR24b4	0.25	No	ns	>0.9999
PpR24a1 vs. PpR24b4	0.25	No	ns	>0.9999

Table C.12: Complete pairwise differences of isolates PpR24, PpR24a1, PpR24b4, and control for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment for unknown compound. Kruskal-Wallis test p-value = 0.857.

Dunn's multiple comparisons test	**Mean rank diff.**	**Sign.?**	**Summary**	**Adjusted P-Value**
Control vs. PpR24	-3	No	ns	>0.9999
Control vs. PpR24a1	-1.75	No	ns	>0.9999
Control vs. PpR24b4	-2.25	No	ns	>0.9999
PpR24 vs. PpR24a1	1.25	No	ns	>0.9999
PpR24 vs. PpR24b4	0.75	No	ns	>0.9999
PpR24a1 vs. PpR24b4	-0.5	No	ns	>0.9999

Appendix D

Chapter 6 Additional Data

Table D.1: Complete pairwise differences for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment for *Orius laevigatus* topical exposure to PpR24. Kruskal-Wallis test p-value = 0.0004.

Dunn's multiple comparisons test	**Mean rank diff.**	**Sign.?**	**Summary**	**Adjusted P-Value**
No-Treat. vs. PBS	-4.15	No	ns	0.8563
No-Treat. vs. WT	-14.9	Yes	***	0.0004
PBS vs. WT	-10.75	Yes	*	0.017

Table D.2: Complete pairwise differences for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment for *Macrolophus pygmaeus* topical exposure to PpR24. Kruskal-Wallis test p-value = 0.0734.

Dunn's multiple comparisons test	**Mean rank diff.**	**Sign.?**	**Summary**	**Adjusted P-Value**
No-Treat. vs. PBS	4	No	ns	0.4358
No-Treat. vs. WT	-2.2	No	ns	>0.9999
PBS vs. WT	-6.2	No	ns	0.0719

Table D.3: Complete pairwise differences for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment for *Aphidius colemani* topical exposure to PpR24. Kruskal-Wallis test p-value = 0.0274.

Dunn's multiple comparisons test	**Mean rank diff.**	**Sign.?**	**Summary**	**Adjusted P-Value**
No-Treat. vs. PBS	-1	No	ns	>0.9999
No-Treat. vs. WT	-9.5	Yes	*	0.0439
PBS vs. WT	-8.5	No	ns	0.0868

Table D.4: Complete pairwise differences for Kruskal-Wallis chi-squared and Dunn's post-hoc test with Bonnferroni adjustment for *Aphidius colemani* mummy topical exposure to PpR24. Kruskal-Wallis test p-value = 0.005.

Dunn's multiple comparisons test	**Mean rank diff.**	**Sign.?**	**Summary**	**Adjusted P-Value**
Control vs. PBS	12.35	Yes	**	0.0044
Control vs. WT	3.85	No	ns	0.9641
PBS vs. WT	-8.5	No	ns	0.0857

Appendix E

Knowledge Exchange

Understanding the impact of phylloplane biocontrol agents on insects

Kristina Grenz, Prof Rob Jackson, Dr Alice Mauchline, Dr Louise Johnson, Prof Mark Fellowes, Dr Georgina Key and Martin Emmett

Introduction

Aphids are a major pest in the agricultural industry. As well as feeding on a wide range of crop plants affecting growth and crop yield, they act as vectors of over 100 plant viruses. Insecticides are widely applied to control aphid infestations but the ability for aphids to develop resistance to chemical treatments and their harmful effect on biodiversity and the environment means alternative methods of control are needed.

Pseudomonas poae

Previous work, conducted by Dr Amanda Hamilton[1] and Dr Deepa Paliwal[2], discovered *P. poae* to be effective at killing aphids. Found on the roots of cabbages, it is capable of survival on the phylloplane. Its virulence is believed to result from two toxicity genes found on its genome and thus far appears to be specific to aphids.

We intend to develop this bacteria as an alternative biological control for use in glasshouses as a foliar spray.

Can we improve the efficacy of the bacteria?

Experimental evolution will be used to see if we can evolve *P. poae* to become better suited to survival on the phylloplane and more effective at killing aphids.

By passaging the bacteria (figure 1) we intend to:

- Improve growth/colonisation on the leaf surface
- Improve perseverance on the plant
- Improve virulence against aphids

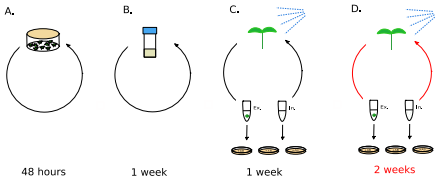

Figure 1. The four methods of bacterial passaging used to attempt to evolve more efficient *P. poae*. **A**. Aphids fed diet inoculated with *P. poae*. After 48 hours recover bacteria from dead aphids and use to inoculate the next passage. **B**. Passage *P. poae* in broth to see if biofilms will form to aid plant surface colonisation. **C**. Spray plants with *P. poae* and recover bacteria from the internal and external leaf surface after 1 week. Count colonies grown on KA + Nitrofurantoin plates to quantify growth success. Use recovered bacteria to spray next passage of plants. **D**. Following the method for C, bacteria will be recovered for colony counts and re-spraying after 2 weeks to assess bacterial perseverance on the plant.

Initial results

Thus far work has focussed on investigating the potential for the bacteria to form biofilms. Biofilms when cells form communities on surfaces that work together for survival. It is potentially extremely useful trait for a biological control[3] as it may improve bacterial survival and persistence on the crop, thus reducing the amount of application and cost of use.

P. poae has shown to be capable of forming biofilms in a broth environment. Although the dataset is incomplete, there are promising indications that attachment strength increases over time (figure 2).

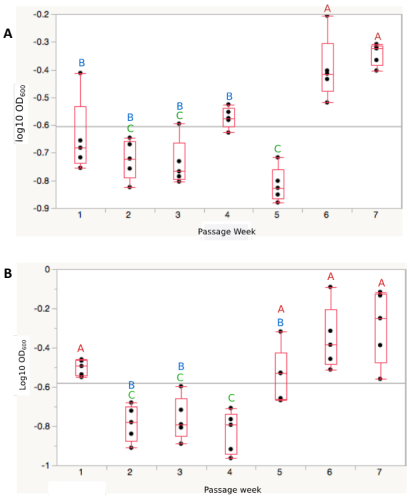

Figure 2. Attachment strength of *P. poae* biofilms at the air-liquid interface in a broth solution. A) log10 OD_{600} over time for 10ul *P. poae* passages and B) log10 OD_{600} over time for 100ul *P.poae* passages. Letters indicate statistically significant different groups.

Next steps

- Finish evolutionary passages and examine trade offs between traits
- Explore if *P. poae* affects the volatiles of the host plant and whether it affects aphid and parasitoid abilities to locate hosts
- Investigate whether *P. poae* has a negative effect on beneficial insects and natural enemies used against aphids, such as parasitic wasps, *Macrolophus* and *Orius* bugs

References:
1. Hamilton, A. (2015) Discovery and development of new phylloplane biocontrol agents to control insect pest. *PhD Thesis*. University of Reading. Reading.
2. Paliwal, D. (2017) Identification and characterisation of new aphid killing bacteria for use as biological pest control agent. *PhD Thesis*. Reading University. Reading.
3. Pandin, C., Le Coq, D., Canette, A., Aymerich, S. and Briandet, R. (2017) Should the biofilm mode of life be taken into consideration for microbial biocontrol agents? *Microbial Biotechnology*. **10** (4) 719-734.

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Figure E.1: Award winning poster at the 2017 AHDB Crops PhD Conference.

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REVIEWS

Endophytes vs tree pathogens and pests: can they be used as biological control agents to improve tree health?

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Abstract Like all other plants, trees are vulnerable to attack by a multitude of pests and pathogens. Current control measures for many of these diseases are limited and relatively ineffective. Several methods, including the use of conventional synthetic agro-chemicals, are employed to reduce the impact of pests and diseases. However, because of mounting concerns about adverse effects on the environment and a variety of economic reasons, this limited management of tree diseases by chemical methods is losing ground. The use of biological control, as a more environmentally friendly alternative, is becoming increasingly popular in plant protection. This can include the deployment of soil inoculants and foliar sprays, but the increased knowledge of microbial ecology in the phytosphere, in particular phylloplane microbes and endophytes, has stimulated new thinking for biocontrol approaches. Endophytes are microbes that live within plant tissues. As such, they hold potential as biocontrol agents against plant diseases because they are able to colonize the same ecological niche favoured by many invading pathogens. However, the development and exploitation of endophytes as biocontrol agents will have to overcome numerous challenges. The optimization and improvement of strategies employed in endophyte research can contribute towards discovering effective and competent biocontrol agents. The impact of environment and plant genotype on selecting potentially beneficial and exploitable endophytes for biocontrol is poorly understood. How endophytes synergise or antagonise one another is also an important factor. This review focusses on recent research addressing the biocontrol of plant diseases and pests using endophytic fungi and bacteria, alongside the challenges and limitations encountered and how these can be overcome. We frame this review in the context of tree pests and diseases, since trees are arguably the most difficult plant species to study, work on and manage, yet they represent one of the most important organisms on Earth.

Keywords Endophytes · Biological control · Trees · Pathogen · Pest · Disease

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Introduction

Importance of trees and their diseases

Being some of the largest organisms on Earth, trees in forest and woodland settings cover 40% of the Earth's terrestrial surface (Fao 2010). This forms a major part of the global biomass and provides habitat for large numbers of animal and plant species with varying levels of association. To humans, the importance of trees for food, timber and non-timber resources has been historically and widely identified (Cazorla and Mercado-Blanco 2016). Carbon sequestration is one of the most significant ecosystem services provided by trees, with

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total carbon stocks in UK forests (including soil) alone calculated at 800 megatons (Quine et al. 2011).

The economic value of trees and shrubs in urban landscapes has been increasingly recognised since the turn of the millennium, with the popularisation of electronic tools for estimating this value, e.g. i-Tree software, and research revealing a suite of functional benefits; providing habitat to urban wildlife, reducing air pollution, intercepting rainfall, shading and reducing heat absorption by man-made surfaces (Binner et al. 2017; Nowak 2004; Tyrväinen et al. 2005; Xiao and Mcpherson 2002). The cultural services provided by trees are also significant, offering both physical and mental health benefits. The capitalised value of the social and environmental benefits provided by woodlands and forests in the UK alone was estimated to be over £29 billion (Lawrence et al. 2009; Willis et al. 2003).

However, trees in particular can be susceptible to attack from pests and diseases (Table 1), especially if invading from other geographical locations (Hansbrough 1965; Tubby and Webber 2010). Most diseases are caused by microbial pathogens (fungi, bacteria and viruses), the effects of which are amplified during periods of unfavourable environmental conditions including unseasonal temperature shifts and extremes in rainfall patterns (Cazorla and Mercado-Blanco 2016) and anthropogenic climate change (Dukes et al. 2009; La Porta et al. 2008; Sturrock 2012; Sturrock et al. 2011; Tubby and Webber 2010).

Tree pest invasions are also increasing alongside climate change and expanded global trade and may act in tandem with native or invasive diseases, as vectors or co-occurring on hosts, to greatly reduce the populations of particular tree species (Brasier 2008; Tubby and Webber 2010), with the potential to ultimately cause their local extinction.

Plant susceptibility to pests and diseases is often related to the stress level of the individual. Unfortunately, trees in urban areas, which have a particularly high value to humans, often face high stress levels. In urban areas, stress can arise from mismatching of the planting stock's ecological traits to the planting site, root deformities, damage and desiccation, planting at improper depths in unsuitable soils, poor nutrient and water availability, and increased exposure to pollutants, xenobiotics and contaminants (Aldhous and Mason 1994; Ferrini and Nicese 2002; Gilman et al. 2015; Grossnickle 2005; Pauleit 2003; Percival et al. 2006; Pfeiffer et al. 2014; Sjöman and Busse Nielsen 2010). Monocultures also pose a specific problem, as plants grown in monoculture are more susceptible to pest and disease outbreaks and are sensitive to changes in climate, which are less likely with polycultures (Sjöman et al. 2012). Lax biosecurity, including the importation of planting stock and tree products, can also drive biological invasions by tree pests and diseases, as has been demonstrated in Europe (Brasier 2008; Epanchin-Niell 2017; Potter et al. 2011). Some non-native pests are highly destructive and can cause substantial damage to forests and urban/suburban trees (Aukema et al. 2011). Such invasions often lead to significant changes in forest structure and species composition, which in turn lead to changes in ecosystem functions (Lovett et al. 2016). Given the range of pests and diseases that trees are facing, the long generation time of trees, the practical difficulty of working with many of them, and also the speed with which the environment is changing, we are faced with a very difficult challenge – how do we improve our disease and pest management to help trees survive?

Classical control approaches for tree pests and diseases

The application of plant protection products (PPPs) for the control of tree pests and diseases is already often limited by ecological concerns and modulated by the particular local context, as exemplified by the varied management of oak processionary moth, *Thaumetopoea processionea*., in Europe (Tomlinson et al. 2015). However, PPPs are well accepted within commercial tree fruit production and the tree care industry of North America.

Presently, PPPs are generally synthetic chemicals that disrupt the cellular function, or life cycle of the target organism. Other PPPs work on a physical basis e.g. killing insect or acarid targets on contact via suffocation, or abrasion of the exoskeleton and subsequent desiccation. These products are typically those formulated for use in agriculture. Aboveground and external tree pests and diseases are often controlled with aqueous sprays of PPPs to the foliage and bark. Specialised high-pressure spray systems can be used for such applications to large trees (Hirons and Thomas 2018).

Internalised pests and diseases, such as nematodes, are more difficult to reach due to their physical concealment within the host; adjuvants (additives) may improve the penetration of externally applied PPPs for such targets e.g. through bark (Garbelotto et al. 2007),

Table 1 Examples of some current major pathogens and pests of trees

Pathogen	Host	Symptoms	Reference
Oomycete *Phytophthora ramorum*	*Larix* spp. and *Quercus* spp. (sudden death)	Shoots and foliage can be affected. Visible as wilted, withered shoot tips with blackened needles. Trees with branch dieback can have numerous resinous cankers on the branches and upper trunk. It has killed millions of native oak and tan-oak trees in the USA	Davidson et al. (2003)
Fungus *Ceratocystis fagacearum*	*Quercus* spp. (wilt)	Symptoms vary between oak species. White oaks may suffer from scattered dieback in the crown to the death of a single limb of major fork. Red oak succomb to the disease usually within a month. Early foliar symptoms start as vein banding whch later develop to foliar necrosis. Thus far only recorded cases in the USA.	Juzwik et al. (2008) Yang and Juzwik (2017)
Fungus *Ceratocystis platani*	*Platanus* spp.	Wound coloniser causing cankers, xylem staining and restriction of water flow throughout the tree resulting in eventual death of the tree. In oriental plane, *Platanus orientalis*, parts of the crown can suddenly die. Can be identified by cankers on the trunk, defined by bluish-black to reddish-brown discolouration of sap-wood and necrosis of the inner bark. Found in the United states and across Europe, such as in Greece, France and Turkey.	Ocasio-Morales et al. (2007) Lehtijarvi et al. (2018)
Fungus *Hymenoscyphus fraxineus*	*Fraxinus* spp. (Chalara ash dieback)	*Dark brown/orange lesions on leaves, diamond-shaped lesions may occur on stems which, if girdled, can cause wilting. The wood beneath lesions usually is strongly stained. Dieback can be seen throughout the crown, with dieback shoots and twigs at the edges of crowns. Originating in Asia but a serious pathogen across Europe.*	*Landolt* et al. *(*2016*)* *McMullan* et al. *(*2018*)*
Fungus *Cryphonectria parasitica*	*Castanea* spp. (blight)	Attacks the bark by entering cracks or wounds which may lead to crown dieback. Discolouration of the bark and dead bark forms sunken cankers. Pin-head sized, yellow-orange pustules develop on the infected bark and exude long, orange-yellow tendrils of spores in moist conditions. Pale brown mycelial fans may be found in the inner bark. Discolouration of the bark may be more visible in younger trees. Widespread thoughout the eastern US, China, Japan and many European countries with an abundance of sweet chestnut.	Rigling and Prospero (2017)
Fungus *Ophiostoma ulmi* and *O. novo-ulmi*	*Ulmus* spp. (Dutch Elm Disease)	Symptoms emerge in early summer as clusters of wilting/yellowing leaves that turn brown and fall. A mixture of healthy and suffering foliage may be seen as the disease progresses throughout the tree. Affected shoots die back from the tip and twigs may turn down-wards. Exposing the outer wood on symptomatic twigs should reveal dark brown or purple streaks. When cut across, a dark brown stain may be present in the outer wood. Common across Europe, North America and Western Asia.	Brasier and Buck (2001)
Fungus *Rigidoporus microporus*	*Hevea* spp. (white root rot)	Fungal mycelium can be found on the tree collar. Multi-layered fruiting bodies form at the base of the tree and white/white-brown rhizomorphs can be seen on the root surface. Off season flowering may occur as well as yellow-brown discolouration of the foliage. Significant funal pathogen to timber and rubber industry in Indonesia, Malaysia, Sri Lanka and the Ivory coast.	Siri-udom et al. (2017) Khairuzzaman et al. (2017) Hamidson and Naito (2004)
Fungus *Colletotrichum acutatum*	*Olea* spp. (anthracnose)	Fruit rot. Soft to dark brown rot that produces an orange, gelatinous matrix in moist conditions and	Talhinhas et al. (2011)

Table 1 (continued)

Pathogen	Host	Symptoms	Reference
		mummification in dry as the fruit loses moisture. In spring there may be extensive yellowing of the leaf blade which in summer leads to premature fall of infected leaves. Found in the majority of olive growing countries.	Cacciola et al. (2012)
Fungus *Cytospora chrysosperma, Phomopsis macrospora,* and *Fusicoccum aesculi*	*Populus* spp. (canker)	Young twigs form brown, sunken, rough circle areas in the bark which may spread to the larger branches. Large cankers may form on the branches an trunk. Orange/orange-brown discolouration of bark is often seen exuding orange-brown viscouse liquid. Fruiting bodies in the bark make the canker appear pimpled. In later stages of infection, perithecial stroma form in the dead cankered areas. Widespread across North America, Europe and China.	Ren et al. (2013)
Fungus *Heterobasidion spp.*	Conifers and some deciduous trees	Symptoms may vary depending on the pathogen involved and host plant. White root rot fungus that in early stages of growth causes staining and discolouration of the host wood. Initial decay is usually pale yellow, developing to light brown and resulting in a white pocket rot with black flecks. Eventually results in tree death. Widespread across the Northern Hemisphere and cases in Australia.	Asiegbu et al. (2005) Garbelotto and Gonthier (2013)
Fungus *Dothistroma septosporum* and *Dothistroma pini*	Conifers (Dothistroma (red band) needle blight)	Yellow bands on needles develop into red bands, where small, black fruiting bodies can occur. Can cause needle dieback, defoliation and eventual tree death. Occurs worldwide. Severe cases in Southern hemisphere plantations of New Zealand, Australia, Chile and Kenya. Also found in North America, Canada and Europe.	Schneider et al. (2019) Barnes et al. (2004) Bradshaw (2004)
Bacterium *Xylella fastidiosa*	*Vitis* spp., *Citrus* spp., *Olea* spp. and several species of broadleaf trees	Leaf scorch/browning, wilting foliage and withering of branches. In extreme cases can result in dieback and stunted growth. Cases found in the Americas, Taiwan, Italy, France and Spain.	Simpson et al. (2000) Araújo et al. (2002) Almeida et al. (2019)
Most likely a decline syndrome with possible Bacterial pathogen components: *Brenneria goodwinii*, *Gibbsiella quercinecans, Rahnella victoriana*	*Quercus* spp. (Acute oak decline)	Stem bleeds occur on the trunk, weeping dark, translucent liquid. Bark cracks, which may reveal underlying dark, necrotic tissue. Lesions and 'D' shaped exit holes of *Agrilus biguttatus* may be present in the bark.	Denman et al. (2014)
Chronic oak dieback – Complex disorder or syndrome (also referred to as oak decline, dieback-decline)	*Quercus* spp. (particularly *Q. robur*)	Results from a combination of abiotic and biotic factors. Early foliage deterioration, gradual branch death and dieback in the crown. Abiotic stressors and weakening of trees allows for opportunistic attack from insects and disease which can result in tree death. Seen in the UK and across Europe.	Thomas et al. (2002) Gagen et al. (2019) Mitchell et al. (2019)
Bacterium *Xanthomonas citri* subsp. *citri*	Citrus cultivars (canker)	Distinct raised, necrotic lesions on fruits, stems and leaves. As the disease progresses, lesions on the stem can appear as corky, rough, dead tissue with a yellow halo. Present in South America, Africa, Middle East, India, Asia and South Pacific.	Graham and Leite (2004) Ference et al. (2018)
Bacterium *Erwinia amylovora*	Pome trees and rosaceous plants (fireblight)	Affects all above ground parts of the plant. The floral recepticle, ovary and peduncles turn a greyish green, eventually whithering to black. Creamy white bacterial droplets may emerge from affected tissues in humid conditions. Shoots wilt rapidly, forming 'Shepard's crooks', that turn necrotic. In later stages, bark becomes	Mohan and Thomson (1996) Johnson (2015) Schropfer et al. (2018)

Table 1 (continued)

Pathogen	Host	Symptoms	Reference
		cracked, sunken and may leak amber bacterial ooze. Found across North America, Central Europe, Israel, Turkey Lebanon and Iran.	
Bacterium *Candidatus Liberibacter* spp.	Citrus trees (Huanglongbing disease)	Blotchy, asymmetric mottling of newly mature leaf blades. Fruit may exhibit stunted growth, premature drop and low soluble acid content. Found across Asia, America and Africa.	Kalyebi et al. (2015)
Bacterium *Pseudomonas syringae* pv *aesculi* (*Phytophthora cactorum* and *Ph. plurivora*)	*Aesculus hippocastanum* (Bleeding canker of Horse Chestnut)	Rusty-red/brown/black gummy ooze found on the bark. Dead phloem under the bleeds which may appear mottled orange-brown. In extensive cases where affected areas encircle the trunk or branch, leaf yellowing and defoliation may occur and eventual crown death. Fungal bodies may also be seen in areas of dead bark. Found across the UK and Europe, including France, Netherlands, Belgium and Germany.	Webber et al. (2008) Green et al. (2009) Green et al. (2010)
Asian longhorn beetle *Anoplophora glabripennis*	Wide range of broadleaved trees	Adults are about 20-40mm long, black with white makings and long, black and white antennae. Oval shaped pits on scraoed into the bark where eggs are laid, occasional sap may be visible bleeding from the damaged areas. Galleries in bark may be up to 10mm in diamteter and several cm long. Wood shavings may be found in distinctive chambers where pupation occurs. Large, circular exit holes from emerging adult beetles in the upper trunk and branches, usually 10mm in diameter. Piles of sawdust may be found at the base of infested trees. Originally from China and the Korean peninsula, now found in the USA, Italy and across the EU.	McKenna et al. (2016) Haack et al. (2010)
Beetle *Dendroctonus micans*	*Picea* spp.	Resin bleeding on stems with resin tubes coloured purple-brown with bark particles where the female enters the trunk. Attacks may occur anywhere on tree. Found across Europe.	Yaman et al. (2010) Wainhouse et al. (1990)
Leaf miner *Cameraria ohridella*	*Aesculus* spp.	In early summer, elongated blotches appear white at first but turn brown throughout the foliage. Caterpillars or pupal cocoons may be seen within mined areas. Heavily infested trees may drop their leaves prematurely. Spread throughout central and eastern Europe.	Pocock and Evans (2014) Gilbert and Tekauz (2011)
Lappet moth *Dendrolimus pini*	*Pinus* spp.	Needle defoliation and subsequent tree death. Prescence of cocoons on trunk. Native to Europe, Russia and Asia.	Ray et al. (2016)
Oak processionary moth *Thaumetopoea processionea*	*Quercus* spp.	Voraciously feed on the foliage of oak trees. Large populations lead to significant defoliation, making the tree susceptible to other threats. Found in Central and Southern Europe. In the UK, outbreaks are localised to London and a few neighbouring counties.	Freer-Smith et al. (2017) Quero et al. (2003)
Ambrosia beetle (Black timber bark beetle), *Xylosandrus germanus*	Wide range of hardwood host species	Infestations can be indentified by entry holes into the bark and distinctive, compact cylindrical frass about 3-4cm in length. Other indications of their presence include wilting and yellowing of the leaves, defensive sap production and dieback. Native to East Asia but has spread across North America, Europe and the Caucasus region.	Agnello et al. (2015)
Citrus longhorn beetle *Anoplophora chinensis*	Deciduous and shrub species	Adult males are about 21mm long, females 37mm. They are black with white markings, with distinctive, long antennae. Symptoms include feeding damage from adult beetles on bark and twigs, circular exit holes in bark and 'T' shaped oviposition slits where eggs are laid within the bark tissue. Tunnelling in bark and larval galleries may cause structural weaknesses, disrupt the	Eschen et al. (2015) Eyre et al. (2010) Haack et al. (2010)

Table 1 (continued)

Pathogen	Host	Symptoms	Reference
		vascular system and result in eventual plant death. Native ranges of China, Japan and Sout East Asia although incidences have occurred in Europe, such as in the Netherlands, Germany, France, Italy, Croatia, Switzerland and the UK.	
Oak Pinhole Borer *Platypus cylindrus*	*Quercus* and other hardwood species	Adult beetles are blackish in colour, 5-7mm long. Usually establishing in stressed trees, galleries about 1.6mm wide are made in the bark with bore dust appearing pale and fibrous. The beetles introduce ambrosia fungi for their nourishment, principly *Raffaelea* spp., which stains the surrounding wood blacky-brown. Found across Europe and North Africa with some incidences occuring in healthy Portuguese trees.	Belhoucine et al. (2011) Bellahirech et al. (2016) Inácio et al. (2011)
Pine wood nematode *Bursaphelenchus xylophilus*	*Pinus* spp. (wilt disease)	Discolouration of some/many branches from green to yellow. Rapid loss of resin flow occurs in 48 hours. Found in Japan, China, Korea, Taiwan, Portugal and Spain.	Futai (2013) Odani et al. (1985)

although many products will still not be transported significantly within the tree. Some PPPs can be injected into the vascular system at the base of the tree and transported upwards e.g. emamectin benzoate used in the control of Emerald Ash Borer larvae in North America (Smitley et al. 2010). The neonicotinoid compound imidacloprid is a soil applied insecticide that is taken up through roots and into the whole plant. However, neonicotinoids face severe restrictions on their use in many countries due to associations with negative impacts on bees (Goulson 2013). Control of fully internalised diseases of trees are also a particular issue, for instance, one of the difficulties in controlling *Verticillium dahliae* and *Xylella fastidiosa* in olive (*Olea europaea*) and grapevine (Baccari and Lindow 2011) is due to the inaccessible location of the pathogen within the vascular system (Cazorla and Mercado-Blanco 2016). Similar difficulties are faced in the control of Huanglongbing disease, *Candidatus liberibacter* spp., which causes citrus greening and is a phloem-limited phytoplasma spread by insect vectors (Abdullah et al. 2009).

Root and soil-borne pathogens have been treated by injections into the soil of PPPs or sterilizing agents such as phenolic compounds or methyl bromide gas (Martin 2003; West and Fox 2002). While many synthetic PPPs break down quickly when exposed on stems or foliage, soil applied compounds may persist for extended periods once bound to soil particles (Edwards 1975).

Stump treatments, e.g. urea, sodium borate, or the saprobic fungus *Phlebiopsis gigantea*, have also been applied to exclude and reduce the build-up of fungal pathogens that can also utilize buried dead wood saprobically, often *Heterobasidion* spp., but may also exclude *Armillaria* spp. and other basidiomycetes, while allowing non-pathogenic species to proliferate (Nicolotti and Gonthier 2005; Nicolotti et al. 1994; Vasiliauskas et al. 2004).

In Europe, and elsewhere, environmental concerns have fuelled a movement away from synthetic "chemical" PPPs or those based on toxic heavy metals e.g. copper (Lamichhane et al. 2018). In the absence of other effective controls this reduction in authorised pesticides may conflict with protecting vital resources such as food and timber.

Biocontrol agents (BCAs)

An area that is gaining much more attention in recent years is biological control (or biocontrol) – the use of biological agents to counter a pest or disease. The desired outcome of a biological control application is to reduce the pathogen or pest population below a threshold of ecological and economic impact, ideally enabling the host to regain health and eventually restoring the invaded community to the pre-invaded state (Bale et al. 2008). This approach is highly favourable because most BCA source species are already present in the host's environment, and in some cases provide a narrow range

of target specificity, so are less likely to be harmful to non-target organisms. BCAs can come in many forms, from viruses or bacteriophage, to bacteria or fungi, and even higher organisms like nematodes, mites or insects (Lenteren et al. 2018).

As PPPs, BCAs are generally applied in similar ways to synthetic compounds and the selected application method typically aims to maximise contact with the target organism. *Bacillus subtilis* QST 713 is a commercialized bacterial strain used in biocontrol programmes around the world (Abbasi and Weselowski 2014). For foliar pathogens like *Botrytis* of fruit or nut trees, *B. subtilis* QST 713 is applied as an aqueous spray, whereas for protection against *Phytophthora* root rots it is applied as an aqueous drench, e.g. via pressurized soil injection systems or irrigation. BCAs may also be physically incorporated into soils (Abbasi and Weselowski 2015). For example, *Trichoderma* strains, often grown on a solid food source such as grain, but also as spore powders, are variously mixed into the soil around roots or placed in cores in close proximity to roots for the treatment of root diseases (Srivastava et al. 2016). One study demonstrated that trunk injections of various *Bacillus* strains into the vascular system of Avocado trees, *Persea americana*, reduced the disease severity of *Phytophthora cinnamomi* infections (Darvas and Bezuidenhout 1987). However, as with the majority of studies discussed in this review, this control method does not appear to have been commercialized or widely utilized to date.

Nematodes, which are used against slugs and snails or insect larvae feeding on roots within the soil, may be dispersed in water and applied to the target area as a drench. Although relatively understudied, nematodes and other soil microfauna e.g. springtails, also have potential in the integrated control of soil borne fungal plant pathogens (McGonigle and Hyakumachi 2001, Riffle 1973, Tomalak 2017). Control of stem boring *Zeuzera pyrina* larvae has been demonstrated by injecting nematode suspensions into the stem cavities created by the larvae (Ashtari et al. 2011). The spores of *Verticillium* strain WCS850 have been applied to Elm trees (*Ulmus* spp.) via punctures in the bark of the tree to induce host resistance to Dutch Elm Disease, caused by the pathogens *Ophiostoma ulmi* and *O. novo ulmi.* The BCA itself does not move far from these sites and the disease is controlled via plant-mediated effects (Scheffer et al. 2008).

Natural enemies are also a popular option for biological control of insect pests in agroforestry settings (Dix et al. 1995). Insects as BCAs have shown great applicability for controlling pests of woody plants, forming around 55% of such introductions up until 2010. The establishment rates of natural enemies and success rates were higher when targeting pests of woody plants than other pests (Kenis et al. 2017). Aphids cause extensive economic losses around the world, as one of the major pest groups of crops plants but are also problematic for trees. To control and counter this, aphid predators, including ladybird larvae, lacewings and gall midges as well as adult spiders, carabids (Carabidae) and rove beetles (Staphylinidae) are used in integrated pest management strategies (Evans 2009; Gardiner & Landis 2007; Messelink et al. 2013; Schmidt et al. 2004; Snyder & Ives 2003). However, the efficiency of control is limited due to insufficient post-application persistence, slow kill rate and high host specificity, in combination with generally high production and maintenance costs, and thus contribute to restricted use in pest control.

The greatest challenges of using BCAs with trees, however, relate to the scales associated with trees – many are very large, thus restricting access to the whole tree and canopy, and woodlands can occupy great areas. Arguably, there is also a dearth of information on many tree diseases and pests, especially for newly emergent outbreaks where monitoring endeavours are struggling to keep up with the incidence and speed of outbreaks (Boyd et al. 2013).

Other major challenges in developing BCAs are the identification, characterisation, formulation and application of the agents. Laboratory analyses may not be reliable predictors of the protective capability of biocontrol agents. For example, the modes of action for most BCAs are still not fully understood, and there is no efficient and effective screening method for identifying field-competent BCAs by laboratory tests (Parnell et al. 2016). The development of appropriate screening methods for BCAs may therefore rely on studies of their interaction with plants, which would slow the screening process. Factors affecting production and delivery of a BCA from laboratory to field include loss of viability, storage stability, environmental conditions, compatibility with other microorganisms, and consistent efficacy over multiple time periods including seasonal variations (Bashan et al. 2013; Slininger et al. 2003). In comparison to synthetic PPPs, storage requirements for preserving BCA product efficacy can be far more varied and

particular, which has been a major issue historically (Bashan et al. 2013; Corkidi et al. 2005).

In this review, we will examine the options for using BCAs for tree diseases and consider how they might be used. In particular, we will focus on endophytes, which are a relatively understudied group. Presented here are examples of endophytes reported as biological control agents in the literature, and most have not been commercialized, but have proven effects under laboratory conditions.

Endophytes as BCAs

Endophytes are defined as microorganisms that accomplish part of their life cycle within living host tissues without causing apparent damage to the plant (Schulz and Boyle 2005; Sun et al. 2014). In all ecosystems, many plant parts are colonized by endophytes (Brundrett 2002; Mandyam and Jumpponen 2005). Depending on the species and the interaction, endophytes may be located in roots, leaves or needles, shoots, or adapted to growth within the bark (Grünig et al. 2008; Rodriguez-Cabal et al. 2013; Sokolski et al. 2007; Verma et al. 2007). Endophytes may grow inter– and intra– cellularly as well as endo– and epi– phytically (Schulz and Boyle 2005; Zhang et al. 2006). However, endophytes can switch their behaviour depending on a set of abiotic and biotic factors, including the genotypes of plants and microbes, environmental conditions, and the dynamic network of interactions within the plant biome (Hardoim et al. 2015; Schulz and Boyle 2005).

Plant ecosystems rely heavily on their microbial communities to optimise health (Pfeiffer et al. 2014), though this intimate association can be a fine balance between mutualism and disease (Knief 2014). Microbes (as epiphytes) can colonise the surfaces of roots (rhizoplane) and leaf/shoot (phylloplane) as well as the internal spaces of plants (as endophytes), with overall abundance being higher for epiphytes compared to endophytes, and rhizosphere compared to phyllosphere (Lindow and Brandl 2003). These differences may reflect the short life span of leaves, nutrient richness in the rhizosphere, and the ability of microorganisms to survive in soil in a dormant state for long periods of time (Vorholt 2012) or due to the physiochemical variations between these two respective environments (Lindow and Brandl 2003).

Endophytes can act in defence against pathogens and disease (Ownley et al. 2004), as well as provide protection or act as deterrents to insect herbivores and nematodes (Breen 1994; Slippers and Wingfield 2007; Vega et al. 2008). However, these defensive properties may not be unanimous to every endophyte-host-pathogen interaction, as shown by Gonthier et al. (2019) where investigations into the protective benefits of ectomycorrhizal fungus *Suillus luteus* in Scots pine (*Pinus sylvestris*) against the fungal pathogens *Heterobasidion irregular* and *Heterobasidion annosum* found that it only reduced host tree susceptibility to *H. annosum*, not both pathogens.

The roles of endophytes in disease and pest resistance are comparatively understudied, but recent work has started to highlight the importance of endophytes, in particular, as an increasingly popular biological control option (Dutta et al. 2014; Gao et al. 2010). Endophytes are also being increasingly recognised as potential controls of significant economic threats such as the invasive spotted lanternfly in North America (Eric et al. 2019).

Isolation and identification of endophytes

Traditionally, the research of endophytes has focussed on identification of culturable fungi and bacteria from plants has involved culturing them from plant tissue on different media. Although successful, it is apparent from the use of culture independent approaches (e.g. metagenomics), that the true diversity and abundance of the endophytic community has not been fully represented or utilized (Bisseling et al. 2009). As a result, it is highly likely that a range of potential candidate organisms with beneficial and exploitable biocontrol capabilities are being overlooked (Moricca et al. 2012; Ragazzi et al. 2001). Slower growing endophytic species are likely to be outcompeted or inhibited in the medium by more rapidly growing species. Other species may be as yet unculturable due to lack of a key growth component, because of an obligate relationship with their host plant for survival or due to a range of environmental parameters. Culture-dependent methods tend to favour the dominant endophytic species, so rarer species that have an irregular existence, are likely to be missed in any sampling effort (Moricca and Ragazzi 2008). However, methods used to isolate, and study endophytes have continued to be improved in light of developments in genetics and genomics. The advancements in next generation sequencing (NGS) has greatly improved the study of endophytes by allowing enormous amounts of genetic sequence data to be processed in parallel at a

fraction of the cost of traditional methods (Knief 2014; Rastogi et al. 2013). Metagenomic analysis employing NGS of whole microbial communities allows much deeper and more accurate DNA sequencing, thus providing insight into the composition and physiological potential of plant-associated microorganisms. NGS reveals both culturable and unculturable endophytes that may be beneficial microbes and appropriate isolation media can then be developed to further study these species of interest (Akinsanya et al. 2015). For example, the presence of endophytic fungi in roots of different plant species in a temperate forest in Japan were identified using NGS (Toju et al. 2013), while whole genome analysis of endophytic microbes has revealed the genetic features that directly or indirectly influence the various bioactivities and colonisation preferences (Kaul et al. 2016). Identification, isolation and characterisation of genes involved in beneficial endophyte-host interactions is critically important for the effective manipulation of the mutualistic association between the two. Endophyte genomic analysis has provided a new tool to pick apart the mechanisms of endophytic associations and to reveal the requisite features needed to inhabit plants. Studies have revealed a wide range of specific genes commonly found across genomes that are important for endophytic lifestyles and symbioses. These include genes coding for nitrogen fixation, phytohormone production, mineral acquisition, stress tolerance, adhesion and other colonization related genes (Firrincieli et al. 2015, Fouts et al. 2008, Kaul et al. 2016, Martínez-García et al. 2015).

Examples of tree endophytes as BCAs

As BCAs, endophytes have diverse mechanisms of action, categorised into direct, indirect or ecological effects (Gao et al. 2010). Endophytes may possess the ability to directly inhibit pathogens by producing antifungal or antibacterial compounds. For example, the endophytic bacterium *Bacillus pumilus* (JK-SX001) is particularly efficient at reducing the infection rate and severity of canker caused by three pathogens (*Cytospora chrysosperma*, *Phomopsis macrospora* and *Fusicoccum aesculi*) in Poplar cuttings. This *Bacillus* strain produces a combination of extracellular enzymes (including cellulases and proteases) and other secondary metabolites that are thought to inhibit the mycelial growth of the pathogen (Ren et al. 2013). When *B. pumilus* (JK-SX001) was applied as a root drench, the bacterial cells migrated from the roots up to the leaves and were reported to also increase host photosynthetic activity and ultimately increase biomass production in the saplings, while suppressing pathogenic activities. These results were promising, but the experiments were performed under greenhouse conditions using fast growing, young cuttings that were sensitive to the canker pathogens. These young trees were easier to inoculate and probably more likely to respond to pathogens and colonisation by endophytes than mature trees.

In another study, the pathogen *Phytophthora meadii*, which causes abnormal leaf fall of rubber trees (*Hevea brasiliensis*), was suppressed using the endophytic bacterium *Alcaligenes* sp. (EIL-2) isolated from healthy rubber tree leaves. In dual cultures, *Alcaligenes* sp. (EIL-2) produced a substance that inhibited hyphal growth of the pathogen. When the endophyte was applied as a foliar and soil drench to one-year old greenhouse plants prior to infection by the pathogen, infection rates were reduced by more than 50% (Abraham et al. 2013). Whilst promising in scope, trials need to be conducted in natural systems to ascertain effectiveness *in situ*.

Species of *Pseudomonas* were the most commonly isolated endophytes antagonistic to the oak wilt fungus, *Ceratocystis fagacearum* (Brooks et al. 1994). When grown in dual culture, these endophytes produced siderophores and/or antibiotic compounds in response to the pathogen. Injecting trees with *Pseudomonas denitrificans* reduced crown loss to *C. fagacearum*, but the response varied depending on what time of year the inoculum was introduced. Seasonal changes affect the physiological state of trees and therefore the availability of nutrients in their vascular system to the introduced endophytes (Brooks et al. 1994), which is likely to influence efficacy of the endophytic BCA.

Berger et al. (2015) compared foliar applications of phosphite, and the endophytes *Trichoderma aureoviride* UASWS and *T. harzianum* B100 on reducing the necrotic area of *Phytophthora plurivora* lesions on oak leaves (*Quercus robur)*. Results showed that given the diffusable nature of phosphite it was able to reduce necrosis on both treated and untreated leaves. However, with UASWS and B100, only untreated leaves showed reduced necrosis suggesting that the interaction was affected by a number of fungal secondary metabolites. However, when applied via trunk injections (endotherapy) a similar endophyte,

T. atroviride ITEC was able to significantly reduce the necrosis size, compared to the control and the phosphite treatment, on 30-year-old beech trees (*Fagus sylvatica*) artificially inoculated with *P. plurivora*. It is clear from this example that the effectiveness of an endophytic BCA is likely to be influenced by the mode of application.

Endophytes may induce such delocalized plant defence reactions, called induced systemic resistance, leading to a higher level of host tolerance toward pathogens (Robert-Seilaniantz et al. 2011; Zamioudis and Pieterse 2012). There is increasing evidence that in the initial stages of endophyte colonisation, interactions between beneficial microorganisms and plants trigger an immune response in plants similar to that against pathogens, but that, later on in the plant growth stage and/or interaction stage, mutualists escape host defence responses and are able to successfully colonize plants (Zamioudis and Pieterse 2012). The shoot endophyte *Methylobacterium* sp. strain IMBG290 was observed to induce resistance against the pathogen *Pectobacterium atrosepticum* in potato, in an inoculum density-dependent manner (Pavlo et al. 2011). The observed resistance was accompanied by changes in the structure of the innate endophytic community. Endophytic community changes were shown to correlate with disease resistance, indicating that the endophytic community as a whole can play a role in disease suppression (Pavlo et al. 2011). Inoculation of white pine (*Pinus monticola*) seedlings with native fungal endophytes reduced disease severity caused by *Cronartium ribicola,* the causal agent of white pine blister rust. The results were temporally persistent suggesting a form of induced resistance. However, the authors did not measure any gene expression or defence pathways to confirm this hypothesis (Ganley et al. 2008).

Inoculation of part of a plant with an endophyte may benefit plants via the production or suppression of phytohormones; for example, genes encoding proteins for biosynthesis of indole acetic acid (IAA) (Zúñiga et al. 2013), cytokinins (CKs) (Bhore et al. 2010) and gibberellins (GAs) (Shahzad et al. 2016) are often present in the metagenome of plant endophytic bacterial communities (Liu et al. 2017). Induction of jasmonic acid biosynthesis enhances localized resistance to biotic agents such as *Hylobius abietis* (large pine weevil) (Heijari et al. 2005), *Ceratocystis polonica* (bluestain fungus) (Krokene et al. 2008; Zeneli et al. 2006) and *Pythium ultimum* (white root rot) (Kozlowski et al. 1999). Mycorrhizae can influence tree susceptibility and tolerance to economically important root pathogens such as *Heterobasidion* spp. and *Armillaria mellea*, even in the absence of direct antagonism of the pathogen by the endophyte (Gonthier et al. 2019; Nogales et al. 2010). Mycorrhizae are well recognized for their positive influence on tree growth and health so may antagonise pathogens via plant-mediated responses or ecologically through inhabiting the same niche, as is seen in other endophytes. The economically important tropical tree, *Theobroma cacao,* is a natural host to endophytes that can significantly reduce the foliar damage caused by a *Phytophthora* species (Arnold et al. 2003). Leaves inoculated with endophytes showed reduced leaf necrosis and mortality when exposed to the foliar pathogen compared to endophyte-free leaf controls. The method of defence appears to be either direct or ecological and not one of induced plant resistance. Only leaves inoculated with the endophytes were resistant to *Phytophthora* infection. This may pose a problem for feasible endophyte application as a BCA if effective disease control is dependent on each individual leaf being sprayed with the endophyte inoculum.

Host-associated microbes can colonize the host horizontally via the environment, vertically from within the parent to the offspring, or by mixed transmission modes (Bright and Bulgheresi 2010). Ecological and evolutionary relationships affect transmission mode and vice versa (Frank et al. 2017). Theory predicts that vertical transmission evolves when symbiotic partners are mutualistic, as a way to ensure faithful transmission of the beneficial symbiont from one generation to the next (Herre et al. 1999). Vertical transmission of bacterial symbionts from parent to offspring is, indeed, common in systems where the symbiont provides an indispensable function, as in the extensively studied nutritional symbioses between bacteria and insects (Moran 2006). Vertical transmission via seeds is also well documented for certain groups of fungal endophytes, e.g., the well-studied *Epichloë* fungal endophytes of grasses (Schardl 2001).

Entomopathogens including fungi, nematodes and bacteria, naturally play important roles in regulating insect populations and are being exploited in biocontrol strategies (Lacey et al. 2015). Miller et al. (2002) investigated the effects of endophytic organisms in white spruce trees (*Picea glauca*) on the pest spruce budworm. They observed that larval growth was significantly

affected by the presence of the endophytes, with some strains proving toxic to the insects. Production of endophytic metabolites is thought to have been the antagonistic factor and in a follow-up study conducted by Miller (2008), the presence of rugulosin toxin produced by the needle endophyte in nursery grown *P. glauca* significantly reduced budworm (*Choristoneura fumiferana*) growth. Decreased palatability for insects and antagonism towards pathogens of needles might be possible benefits for the host trees. Sieber (2007) also found that the colonization of elm bark by *Phomopsis velata* had significant effects on two beetle pests of bark, *Scoltus scolytus* and *Scolytus multistriatus*. These beetles are known vectors of the Dutch Elm disease pathogen *Ophiostoma ulmi* and on introduction of *P. oblonga*, there was a noticeable reduction both in beetle galleries as well as larval success rate thus providing evidence in support of an effective biocontrol agent. In addition to the discovery of more effective isolates and toxins, an increase in the use of entomopathogens will rely on innovations in formulation and better delivery systems.

Challenges in biocontrol of tree pathogens and pests with endophytes

Climate change has and will continue to alter the ranges of pests and diseases and aid their establishment by subjecting plants to stress (Shaw and Osborne 2011). Occurrence of extreme temperatures and weather events, such as heatwaves and flooding, are increasing in frequency worldwide as CO_2 levels increase and thus our natural capital may require active management to protect its current condition (Fischer and Knutti 2015; Hailey and Percival 2015).

Climate change is predicted to have a profound impact on the distribution, abundance, physiology, productivity, phenology, behaviour and ecology of all plant species (Hughes 2000; Nooten et al. 2014). Forest species are particularly susceptible to climate change as the higher longevity of trees hinders rapid adaptation (Broadmeadow et al. 2005; Lindner et al. 2010). Moreover, climate change is known to impact plant-associated microbes some of which play critical, mutualistic roles in maintaining healthy environments. For example, climate change is likely to impact the dispersal of mycorrhizal fungi, key symbionts of trees, which may in turn limit tree migration and colonisation of novel habitats (Pickles et al. 2015). Warming may induce a decrease in arbuscular mycorrhizal fungal colonisation, as has been demonstrated in a manipulated Mediterranean climate, with a likelihood of significant impacts on plant communities and ecosystem function (Wilson et al. 2016).

It has also been recognised that changes in the environment or host can alter the nature of the host-endophyte interaction (Schulz and Boyle 2005). When a tree is subjected to physiological or environmental stress the intimate plant-endophyte relationship is altered and the endophyte may become pathogenic. For example, the fungal endophyte *Discula quercina,* which inhabits healthy *Quercus cerris* trees, causes damage to host structure and function when the tree experiences drought stress (Moricca and Ragazzi 2008; Ragazzi et al. 2001). *Picea abies* (Norway spruce) and other conifers are predicted to become unsuitable for forestry in the central regions of Europe due to rises in temperature (Breymeyer 1996; Fanta 1992), especially at lower elevations (Lexer et al. 2002), which may subject the present large spruce forests to severe stress in the future. Similarly, altered climates may affect BCA function and efficacy. Climate change may also change the lifecycles and feeding behaviour of phytophagous insects, with vector-mediated impacts on tree disease spread (Battisti 2008). It is therefore possible that we will observe an increasing incidence of disease in trees caused by endemic endophytic species, in addition to and potentially interacting with highly destructive pest invasions.

Numerous factors play a role in the under implementation of BCAs for control of diseases in trees and woody plants. These include the size, area, complex root system, inoculum size, and impact of release on the associated ecological system. Many of the difficulties are shared with conventional PPPs, such as reaching internal pests and diseases. Arthropod pests of crops and trees are extremely diverse like their hosts and thus can be notoriously hard to control. Rapid reproduction rates of some of these species (e.g. aphids, gypsy moth, and spruce budworm) mean dense infestations can arise rapidly, reaching levels damaging the plants that then leads to losses that impact both the environment and local and regional economies. Furthermore, different life cycle stages mean that one method of control may not be adequate to manage a pest population effectively, such as in the case of scale insects (Mansour et al. 2017). There is a general consensus that the detrimental effects of insect pests on crops and woody plants are set to worsen with accelerated climate change and control of these will be required for agriculture to keep up with the demands of a growing world population (Dukes et al. 2009).

Effects of introduction of 'alien' species. What are the consequences?

It is as yet unclear as to whether endophytes introduced as BCAs on plants may be effective in reducing disease, but another important aspect is to understand if they have adverse effects on the natural microbial community of the host when the plant is under environmental stress. The introduction of endophytes that have not co-evolved with the host plant may result in the loss of beneficial organisms and so negatively impact the host plant (Whipps 2001). Furthermore, it is important to consider whether the gains provided by the endophyte outweigh the costs associated with it. For example, gall wasps are a problematic species to trees. However, *Apiognomonia errabunda*, the dominant endophyte in beech leaves, has been found to cause abscission of galls by forming necrotic tissue around the affected area, but this may, in time, prove to be more harmful to the host than the gall would have been (Sieber 2007). Furthermore, there is also a risk that some endophytes may not be as useful as hoped in integrated pest management systems as they may affect the efficacy of other BCAs employed to combat pest species. Bultman et al. (2017) found that although endophytes proved effective against plant herbivores, they had repercussions higher up the trophic chain, significantly affecting the performance of parasitoids by reducing pupal mass, which would reduce the parasitoids' success as a BCA.

Pros of using endophytes as BCAs

1. No known adverse environmental effects of native endophytic biological control agents as they are already present in the plant ecosystem.
2. Ability to colonize internal host tissues, the same ecological niche as pathogens, allowing persistence and competition for resources in addition to antagonism. The internal tissue also protects the biological control agent from dangerous UV rays and temperature fluctuations therefore they can persist for longer periods of time.
3. As well as controlling infection, endophytes may simultaneously promote plant growth, for example by increasing photosynthetic activity (Ren et al. 2013).
4. Narrow range of target specificity, less likely to be harmful to non-target organisms.
5. Endophytes may induce systemic resistance in the host and may consequently induce resistance against other pathogens and/or pests (Zamioudis and Pieterse 2012).
6. Pathogens may be less likely to acquire resistance to endophytic BCAs than they are to pesticides due to dynamic interactions.

Cons of using endophytes as BCAs

1. Most research to date has taken place in laboratory conditions, but it is unknown how the endophyte-pathogen interaction will alter in the presence of changing environmental conditions and competition with other organisms in the tree ecosystem.
2. More research must be conducted to find the optimum time for delivery of biocontrol agent inoculum, as seasonal changes in weather and tree physiology could alter efficacy (Brooks et al. 1994).
3. In some cases, resistance to pathogens is isolated only to the plant part that is inoculated with endophytic control. Delivery, and systemic transmission, of BCA to whole tree is likely to be difficult in many cases.
4. Possible changes in host-endophyte-pathogen interaction with climate change, could the endophyte itself become a pathogen? (Moricca and Ragazzi 2008; Ragazzi et al. 2001).
5. Endophytic BCA may alter the microbial community of the host tree, which may adversely affect the host or may have consequences at higher trophic levels.

Conclusion

With growing concern about environmental pollution and the harmful effects of chemicals, the use of biological control as an alternative environmentally friendly option is becoming necessary. The traditional breeding of trees for resistance remains one potential route, but it is a strategy that might be outpaced by the spread and introduction of pests and diseases, as well as being a time consuming and sometimes difficult task. Despite the challenges confronting biocontrol of tree diseases and

pests, research shows that endophyte treatments can be successfully implemented and there is clear potential for endophytes to be applied to trees as BCA in the future. However, it is unclear how the endophyte enters the plant tissues and disperses throughout the plant. The efficacy of the biocontrol method can be enhanced by integrating it with complimentary cultural and environmental conditions to stimulate plant health and enhance inhibition of the pathogen or pest, but this still requires more attention in the future. Advancements in molecular techniques, such as NGS, are revealing more accurate community structures and, as new environments are studied, it is very likely that new bacterial and fungal species will be discovered and enable the dissection of community effects of individual organisms. Application of community analysis and metagenomics technologies in future studies will advance understanding in both plant-microbe associations and biological control science, with endophytes being prime candidates for use as BCAs.

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Compliance with Ethical Standards

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